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UNRESTRICTED

DIAGNOSIS AND CONTROL OF FOOT ROT
PATHOGENS OF WHEAT.

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A thesis submitted in partial fulfilment of the requirements of
the Open University for the degree of Doctor of Philosophy

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ABSTRACT

Foot rot disease of wheat is caused by the pathogens *Fusarium culmorum*, *F.avenaceum* and *Microdochium nivale*. Symptoms of foot rot are a general browning of the stem base and leaf sheath. There is a discrepancy between the ability of fungicides to control these pathogens *in vivo* and *in vitro*, and no relationship between disease symptom severity and yield loss has been established in wheat. The identification of the causal agents of foot rot disease is not possible from examination of disease symptoms alone.

This work showed that the azole fungicides flusilazole and prochloraz inhibited the germination of conidia and mycelial growth of *F.culmorum*, *F.avenaceum* and *M.nivale* *in vitro* to a varying extent. However, no consistent control of these pathogens in wheat was observed in the field using the same fungicides. Further studies employing a semi-controlled outdoor experiment showed a relationship between density and timing of inoculum application, disease symptom severity and yield loss in wheat artificially inoculated with *F.culmorum* and *M.nivale*.

Molecular marker systems were used to address the problem of pathogen detection and identification. A Random Amplified Polymorphic DNA (RAPD) assay was developed to differentiate *F.culmorum*, *F.avenaceum* and two types of *M.nivale* (*M.nivale* var.*nivale* and *M.nivale* var.*majus*) *in vitro*. Selected RAPD products were cloned and sequenced and species specific primers constructed from this sequence information. These primers were used in the polymerase chain reaction (PCR) and were shown to detect the pathogens in host tissue. This technique was adapted by addition of a competitor fragment to the PCR reaction resulting in a quantifiable competitive PCR assay. Using this method the fungal biomass of each pathogen present in the host tissue could be estimated. The development of these techniques for the identification, detection and quantification of *F.culmorum*, *F.avenaceum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* in plant tissue will allow more extensive studies of the epidemiology of these species, the competition between species and the effect of fungicides on these pathogens can be carried out.

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INTRODUCTION

The genus *Fusarium*

According to Booth (1971) the genus *Fusarium* was created by Link (1809) to include species with fusiform non-septate spores borne on a stroma, and the genus was later validated by Fries (1821) who included it in the order Tuberculariae. With the development of pure culture methods for *Fusarium* identification, the essential character of the genus was accepted as the presence of a fusoid macroconidium with a foot cell bearing some sort of heel. This characteristic allowed discrimination between *Fusarium* and *Cylindrocarpon*, the most morphologically similar genus.

Early taxonomic work on *Fusarium* was carried out by Wollenweber & Reinking (1935). These workers began with approximately 1000 named species of *Fusarium* which they then organised into 16 sections which consisted of 65 species, 55 varieties and 22 forms. This separation of species was carried out using morphological characteristics including shape of basal foot cells, length and width of macroconidia and the presence or absence of chlamydospores. This taxonomic system was extremely complicated, and it was therefore difficult to construct a practical identification key. These workers did not use cultures derived from single conidia in their investigations and the effect of cultural variation on classification was therefore not considered.

Snyder & Hansen (1945) pioneered the use of single conidial cultures for *Fusarium* identification, and were able to condense the 16 sections described by Wollenweber & Reinking (1935) into just nine sections using a system based primarily on the morphology of macroconidia. Snyder & Hansen (1945) also showed that due to cultural variation, the progeny of a single parent could be placed under different species and even in different subsections using the method of Wollenweber & Reinking (1935), and therefore concluded that the characteristics used for speciation by these workers were too narrow. *Fusarium* taxonomy is fraught with difficulty, as recognised by Windels

(1991) who stated that the fundamental problem inherent in *Fusarium* identification is that members of the genus vary widely in morphological and non-morphological characteristics including virulence, and it is these criteria that are used in taxonomic systems.

The difficulties in choosing an acceptable method for *Fusarium* identification are illustrated by Nelson (1991), who proposed that the system employed by Wollenweber & Reinking was too complex for the routine identification of *Fusarium* species, and that of Snyder & Hansen (1945) too simple. According to Windels (1991) the development of molecular tools will aid in defining and confirming the boundaries and relationships of sections and species of *Fusarium* taxonomy that are currently based only on morphological characteristics. The use of molecular techniques for the identification of *Fusarium* species and *Microdochium nivale* (Fries) Samuels & Hallet [conidial state of *Monographella nivalis* (Schaffnit) E.Mull., formerly classified as *F.nivale* (Mueller, 1977)] is fully discussed in Chapters 3 and 4. *Microdochium nivale* was previously classified as *Fusarium nivale*, and due to the similarity of the diseases of wheat caused by this pathogen and several of the *Fusarium* species, it has been included in this work. The taxonomy of *Microdochium nivale* and the development of molecular tools to facilitate its identification are described in Chapter 3.

Diseases caused by *Fusarium* species

Fusarium pathogens are known to attack a very wide range of plants, and can also cause major storage rots which produce toxins and contaminate food. Examples of diseases caused by *Fusarium* pathogens include serious wilts such as Panama disease of bananas caused by *F.oxysporum* Schlecht. (Armstrong & Armstrong, 1948), root rots of the broad bean (*Vicia*) and French bean (*Phaseolus*) caused by *F.solani* (Mart.) Sacc., (Burke, 1965; Yang & Hagedorn 1965), cankers of hardwood trees, also caused by

certain strains of *F.solani* (Hocking 1968, Brown 1964) and diseases of the Graminae such as pokkah-boeng of sugarcane, bakanae disease of rice, snowmould of turfgrasses caused by *Microdochium nivale* and pre- and post-emergence blights of cereals caused by the species *M.nivale*, *F.culmorum* (W.G. Smith) Sacc., *F.avenaceum* (Corda ex Fr.) Sacc. (*Gibberella avenacea*) and *F.graminearum* Schwabe. (*Gibberella zeae*) amongst others.

Fusarium species and *M.nivale* can cause diseases of cereals which occur at all stages of crop development. For example, *M.nivale* was shown to cause seedling blight of winter wheat in Eastern England in 1958-59 (Baker 1972), and *F.culmorum* was considered by Cook (1980) to be the main cause of *Fusarium* foot rot of wheat in the Northwestern states of America. *Fusarium* diseases of cereals are usually catagorised as *Fusarium* seedling blight, *Fusarium* foot rot and *Fusarium* ear blight. . Although the disease of interest in this work was foot rot of wheat caused principally by *F.culmorum*, *F.avenaceum* and *Microdochium nivale*, the disease cycles of these pathogens may also involve seedling and ear diseases, and as each disease may provide a possible source of inoculum for the subsequent stage, a brief review of each of the three diseases is given. The disease cycle of *Fusarium* on small grain cereals is illustrated in Fig I 1a.

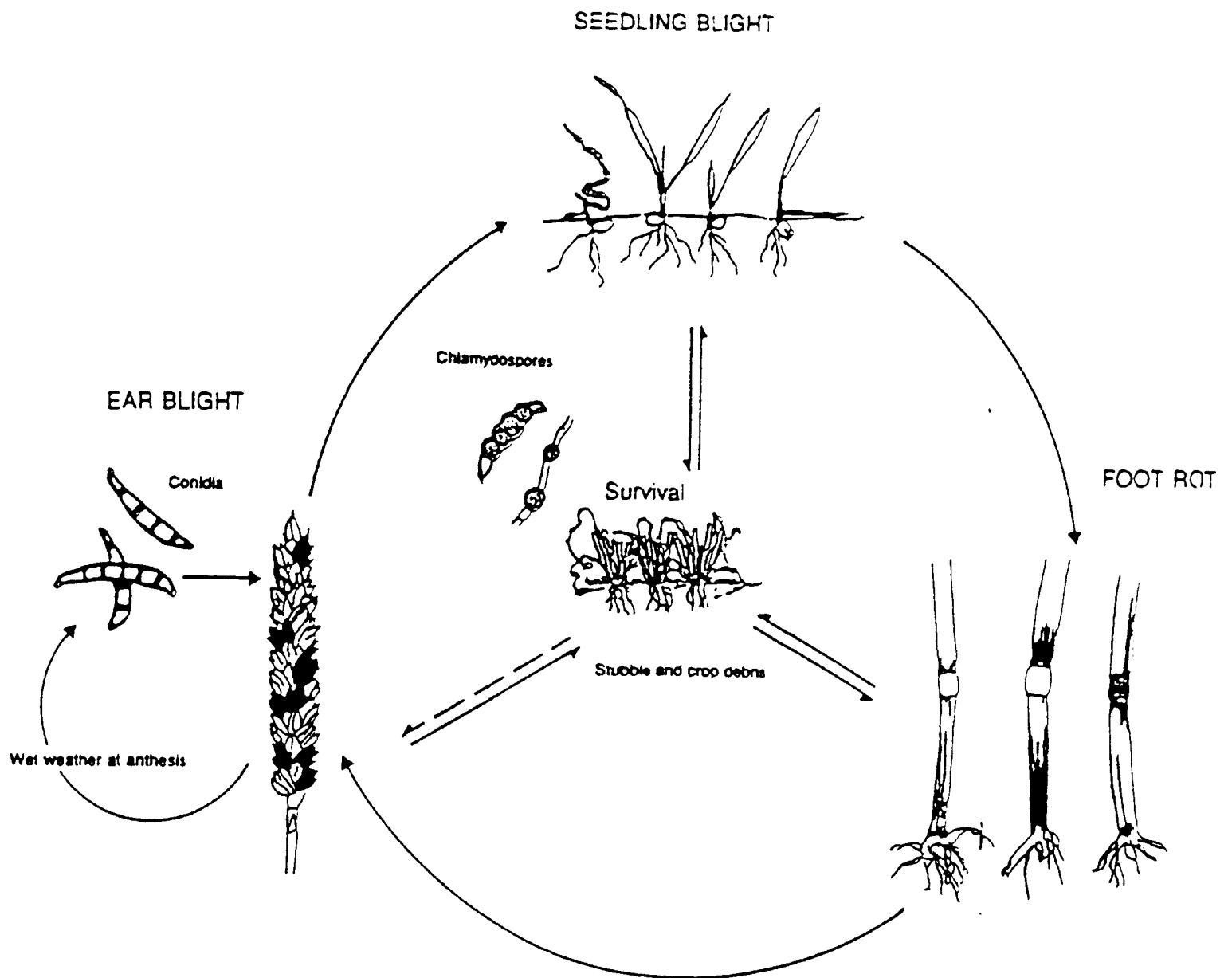


Fig 11a Generalised disease cycle of *Fusarium* on small grain cereals (from Parry *et al.*, 1994)

Seedling diseases of cereals caused by *Fusarium* species and *M.nivale*

Wheat seedlings can be affected by pre- and post-emergence damage caused by seed contaminated with *M.nivale*, *F.culmorum*, *F.avenaceum* and *F.graminearum*, or by infection from soil-borne inoculum of these species. Uoti (1976) showed that when spring wheat and barley seeds were inoculated separately with five different *Fusarium* species, *F.culmorum* was found to be the most destructive, causing severe seedling blight, and that the other species tested (*F.avenaceum*, *F.graminearum*, *F.poaë* (Peck) Wollenweber. and *F.tricinatum* (Corda) Sacc.) had a less marked effect. Symptoms of seedling blight range from death soon after germination to superficial stem lesions on emerged plants. Millar & Colhoun (1969) found that seedlings grown from seed artificially inoculated with *M.nivale* had brown lesions on their roots, coleoptiles and leaves, and produced few tillers early in the season. Such artificial inoculation of the seed with *M.nivale* resulted in severe pre-emergence death (up to 80%), often soon after the germination of the seed. The first and second leaves of infected plants have also been observed to bear lens shaped, pale brown lesions (Noble & Montgomerie, 1956; Millar & Colhoun 1969). The invasion of spring barley seedlings by *Fusarium* species was studied by Perry (1986), who showed that the earliest attacks appeared to occur in the region of the mesocotyl. He also reported that many seedlings were infected without visible symptoms, and suggested that *Fusarium* species frequently behave as endophytes rather than parasites.

When Colhoun & Park (1964) conducted glasshouse experiments with artificially inoculated wheat seeds they found that pre-emergence death of the seedlings caused by *F.culmorum* and *F.graminearum* was most severe in dry soils and increased with increasing temperature. The importance of inoculum potential on the infection and stem lesion development in seedlings by *F.culmorum* and *F.avenaceum* was stressed by Colhoun *et al.* (1968). These workers found that *F.avenaceum* was a much weaker

pathogen than *F.culmorum* and required a much higher seed spore load to achieve the same degree of pathogenicity (Colhoun & Park 1964). Dickson (1923), concluded that soil temperatures less than 12°C during seedling germination prevented *F.graminearum* attacking wheat seedlings whereas higher temperatures favoured disease. He also found that low soil moistures, even at low temperatures were very suitable for the development of disease on seedlings. Colhoun & Park (1964), and Colhoun *et al.* (1968) considered that seedlings in wetter soils tended to escape attack by *F.culmorum* because they developed more rapidly with a sufficient water supply. They showed that soaking seeds before sowing reduced the amount of disease. Kovacikova (1993) found that the viability of spores of *F.culmorum* and *F.avenaceum* was affected differently by temperature. In water saturated soil *F.culmorum* was capable of surviving at lower temperatures than *F.avenaceum* due to the production of chlamydospores by *F.culmorum*. This worker therefore concluded that *F.culmorum*, unlike *F.avenaceum* could provide a source of inoculum in the top layer of soil for the infection of seedlings. Colhoun *et al.* (1968) stressed the importance of interactions between certain factors to cause seedling diseases. According to these workers, the most important factors are soil moisture, temperature and especially level of seed inoculum. For *F.culmorum* on wheat there was little pre- or post- emergence death or stem lesion development at any temperature or soil moisture until the spore load reached fairly high levels (e.g. at least 10⁵ spores/25g seed).

An important method for the control of seedling blight caused by *Fusarium* species and *M.nivale* is the use of fungicide treated seed, which has been shown to be an effective means of reducing seedling disease (Bateman, 1979, Jamalainen, 1962). The use of fungicide treated seed is reviewed more extensively in Chapter 2.0, and is therefore not discussed further here. Other work to control seedling blight caused by the *Fusarium* pathogens and *M.nivale* has included attempts to isolate saprophytes antagonistic to

these pathogens, and to use them in the control of plant disease. Ledingham *et al.* (1949) clearly demonstrated that bacteria on the surface of wheat seed greatly reduced the incidence of seedling disease caused by seed borne fungi. The biological control of *Mnivale* on wheat seed by the use of a naturally occurring antagonistic fungus was demonstrated by Millar & Colhoun (1969), who found that *Gliocladium roseum* controlled *Mnivale* on inoculated wheat seed, but not on naturally infected seed. Tveit & Wood (1955), showed that certain isolates of *Chaetomium cochliodes* and *C.globosum* gave good control of *Mnivale* and were as effective at reducing seedling disease as an organomercury seed treatment, and Bateman (1979) demonstrated that *Alternaria* when dually inoculated on wheat seed with *Mnivale* reduced the severity of subsequent seedling infection. He found that *Alternaria* could also inhibit the colonisation of the ear by *Mnivale* regardless of whether or not the *Alternaria* was inoculated before or after the *Mnivale*. Al-Hashimi & Perry (1986) showed that *Trichoderma viride* a saprophyte on straw completely suppressed the growth of *Mnivale* in culture and also reduced the number of diseased seedlings when added to soil in which seeds with a low level of *Mnivale* inoculum were sown. However, there appear to be no cases reported in which the direct application of antagonists has controlled *Fusarium* disease of cereals under natural conditions in which the introduced organism has to compete with the normal microflora in conjunction with the pathogen itself.

Foot rot of cereals caused by *Fusarium* species and *Mnivale*

Foot rots of cereals can be caused by several species of *Fusarium* including *F.culmorum*, *F.avenaceum*, *F.poa* and *F.graminearum*, and also by *Microdochium nivale*. Bennett (1928) associated a foot rot of wheat caused by *F.avenaceum* and *F.culmorum* with the problem of lodging. Symptoms characteristic of *Fusarium* foot rot

on cereals include a general browning of the stem base tissue. A pinkish spore mass can also sometimes be visible on the lowest internode. During tillering, *Fusarium* or *M.nivale* infection is seen as a general brown discolouration or striping on the outer leaf sheaths at the base of the plant, sometimes developing into a water-soaked foot rot. Severe stem base disease causes death of tillers, and may result in whiteheads occurring at random in a green crop. The stem can be easily broken at the rotted node, and the pink mould of the fungus can be seen. *M.nivale* is also able to cause a disease of cereals known as snow mould. This disease is widespread in areas where crops are covered in snow during the winter, for example in Northern Europe. Affected tissues can be covered by grey or pink mycelium, and leaves, or in severe attacks crowns, can be killed.

Moore (1959) suggested that brown foot rot (mainly associated with *F.culmorum* and *F.avenaceum*) was widely distributed in poorly drained acid soils, but was rarely serious, except in the North of England and Scotland. However, surveys of the incidence of stem base disease of cereals caused by *Fusarium* species and *M.nivale* in the U.K. show that these pathogens are widespread. There is little published data on the incidence of *Fusarium* species on winter wheat in the early stages of crop growth. In surveys undertaken in Scotland by Rennie *et al.* (1983), *M.nivale* was isolated from 80-90% of crops and *F.avenaceum* and *F.culmorum* from 25-45% of crops sampled. Locke *et al.* (1987) recorded a mean of 35.8% wheat tillers infected by *Fusarium* species during a survey in 1986, and 82.5% of these were *M.nivale*. Work by Parry (1990) substantiated these findings. In a three year survey (1987-1989) carried out in the Midlands, U.K., he noted the predominance of *M.nivale* in winter wheat; 65% of shoots sampled in the spring of 1989 were infected with this species.

Cereal foot rots have been shown to occur as a complex of several diseases. Hoare (1987) found that in glasshouse tests *F.culmorum* in combination with *Gaeumannomyces*

graminis (the take-all fungus), caused significantly more stem base damage to plants than *G.graminis* alone. However, in combination with the eyespot fungus *Pseudocercospora herpotrichoides* and *Rhizoctonia cerealis* (sharp eyespot), *F.culmorum* did not cause any additional damage. Parry (pers.comm) suggested that when field plots of winter wheat were inoculated with *F.culmorum* and *P.herpotrichoides* singly, and in combination, the proportion of plants infected with *F.culmorum* increased from 60% in plots inoculated with *F.culmorum* alone, to 90% in plots inoculated with both fungi, ie infection by *F.culmorum* was enhanced by the presence of the eyespot fungus.

Cook (1980) stated that the occurrence of foot rot symptoms caused by *Fusarium* species was correlated with the temperature and water potential most appropriate for the mycelial growth of the fungus. Cook & Christen (1975) showed that *F.graminearum*, and *F.culmorum* all required progressively drier conditions on osmotically adjusted agar media for maximal growth when the temperature of incubation was increased from 10°C to 35°C. They proposed that the responses of the pathogens matched their ecological distribution in the Pacific Northwest of America; *F.graminearum* and *F.culmorum* both caused foot rot of wheat in hot, dry soil, with *F.graminearum* being associated with slightly hotter and drier soil than *F.culmorum*. Similarly, Hargreaves & Fox (1978) found that the survival of spores of *F.avenaceum* was best in dry soils and within the pH range 3.8-4.6. These workers found that temperatures of at least 56°C for 30 minutes were required to eliminate spores from the soil. Cook (1968) first reported severe *Fusarium* foot rot and crown rot in central Washington in the 1960s. Papendick & Cook (1974) related this appearance to severe water stress in semi-dwarf wheats given large amounts of nitrogen. Colhoun (1970) stated that seedlings with lesions caused by *F.culmorum* or *M.nivale* did not develop foot rots later unless the air humidity at the base of the plant was high, irrespective of whether or not the soil was

wet or dry. Booth (1971) considered that *F.culmorum* was a soil-inhabiting necrotroph possessing highly competitive saprophytic ability. The pathogen was able to survive either as chlamydospores in the soil, or as mycelia in plant debris, and was able to survive up to two years on infected straw, remaining viable at a soil depth of up to 50cm, and therefore providing a source of inoculum for the development of foot rot infections.

Clarkson & Polley (1981), conducted an assessment of losses caused by stem base diseases in cereals. Equations relating percentage yield loss to the percentage of stems with moderate or severe disease symptoms were used to estimate national crop losses from assessments carried out in ADAS winter wheat surveys in England and Wales from 1975-80. These workers stated that even though the association between disease and yield loss had not yet been clarified for *Fusarium*, losses due to nodal infection ranged from 0-15 %, to internodal infection from 0-47% and dual nodal/internodal interaction from 0-40%. Polley *et al.* (1991) suggested that *Fusarium* diseases which occurred on both the stem base and ears could affect wheat crop yields to the same degree as *Pseudocercospora herpotrichoides*, the cause of eyespot disease, which alone may have resulted in losses to the 1989 harvest of up to £11.6m despite the application of fungicides. The effect of *Fusarium* species and *Mnivale* on disease severity and yield is discussed fully in Chapters 1 and 2. The control of foot rot of wheat by fungicides is poor, and there are few records of reliable control of this disease in the field. The control of *Fusarium* species and *Mnivale* using fungicide seed treatments and foliar applied fungicides is discussed in detail in Chapters 1 and 2, and will therefore not be described here.

Ear blight of winter wheat caused by *Fusarium* species and *M.nivale*

Fusarium avenaceum, *F.culmorum*, *F.graminearum*, *F.poa* and *M.nivale* can all occur on the ears of cereals and produce symptoms of ear blight (Scott & Benedikz, 1987).

Wong *et al.* (1992) found that *F.graminearum* and *F.culmorum* were the main causal agents of *Fusarium* ear blight in severely affected crops of wheat in Manitoba, Canada.

Stack & McMullen (1985) also found *F.graminearum* and *F.culmorum* to be highly

pathogenic to wheat ears, and Sutton (1982) reported that *F.graminearum* was the principal pathogen causing ear blight in most countries. Water soaked spots on the

glumes of wheat ears provide an early indication of infection by ear blight pathogens.

The glumes of an infected spikelet can become bound together by the growth of fungus within the spikelet, and a pink mass of sporulating fungus is often seen at the base of the glumes. The disease can progress to affect whole spikelets, which then become bleached.

Sutton (1982) reported the association between above average rainfall in the Summer and epidemics of ear blight in Canada. He showed that for *F.graminearum*, persistent wetness or high humidity and temperatures above 15°C were necessary for infection and disease development. Bennett (1933) reported damage of wheat ears by *M.nivale* under moist conditions, and a reduction in the value of the crop partially by yield losses, but mainly by discolouration of the sample. Such infection of wheat seed by *F.culmorum*, *F.avenaceum* and *M.nivale* obviously provides an important source of inoculum for the development of seedling blight when infected seed is sown, and therefore also for the later development of foot rots of cereals. However, the pathogens are able to infect alternative hosts, and have developed survival mechanisms by which inoculum can be carried over from one crop to another. It has also been demonstrated that *Fusarium* pathogens are also able to infect other hosts. For example, Jenkinson & Parry (1994) found that *Fusarium* species pathogenic to winter wheat could be isolated from a range

of common broad-leaved weeds, and Atanasoff (1920) observed an increase in *Fusarium* ear blight in wheat where large populations of weeds were present. Tusa *et al.* (1980) suggested that maize-wheat rotations could also be implicated in an increased incidence of ear blight caused by *Fusarium* species. The removal or ploughing in of crop debris to reduce inoculum potential was shown to significantly reduce the incidence of *Fusarium* ear blight in wheat (Teich & Nelson, 1984).

Work on methods for controlling *Fusarium* ear blight has shown that differences in relative resistance to *Fusarium* ear blight between varieties and wheat genotypes do exist, but not at a level that can be considered useful according to Martin & Johnston (1982) and Mesterhazy (1983). Parry *et al.* (1984) showed that there were significant differences in varietal reactions for field plots of wheat infected with *F.culmorum*, and Miedaner *et al.* (1993) found that resistance of different genotypes of winter rye to infection by artificially inoculated *M.nivale* was expressed under different environmental conditions, and that there was significant genotypic variation for snow mould rating, plant loss rating, number of spikes and grain yield relative to non-inoculated plants. Varietal resistance to *Fusarium* ear blight was reviewed fully by Parry *et al.* (1995a). Parry *et al.* (1995a) in their review of *Fusarium* ear blight reported that fungicidal control of *Fusarium* ear blight is at best inconsistent due to problems with the timing of fungicide applications, the interaction between *Fusarium* species, and reports of widespread resistance to MBC fungicides in populations of *M.nivale* (Locke *et al.*, 1987; Pettitt *et al.*, 1993). However, control of *Fusarium* ear blight has been demonstrated in field (Fehrmann & Ahrens, 1984), and in glasshouse trials (Hutcheon & Jordan, 1992) artificially inoculated with *F.culmorum* and *F.graminearum*, where the fungicide prochloraz was applied. A full review of fungicide efficacy against *Fusarium* ear blight can be found in Parry *et al.* (1995a)

1.0 Effect of a triazole and an imidazole fungicide on the conidial germination, germ tube extension and mycelial growth of *Fusarium culmorum*, *Fusarium avenaceum* and *Microdochium nivale* *in vitro*, and on development of foot rot in the field.

The anomaly between the ability of fungicides to control pathogens *in vitro* and their effectiveness *in vivo* is recognised widely, and is a problem in the case of *Fusarium* diseases of cereals. For example, Klein & Burgess (1987) found that the triazole seed treatments fenarimol and triadimefon caused significant inhibition of the growth of *F. graminearum* in laboratory tests, but failed to provide an effective means of control of the seedborne inoculum which caused whitehead formation and yield loss in wheat. In addition, Celetti & Hall (1987), found high levels of toxicity of the seed treatments maneb, carbathiin and triadimenol to *Fusarium* species *in vitro*, but only a slight effect on crown infection by maneb, and no reduction in the infection of crowns by *Fusarium* with the other fungicides. Polley *et al.* (1991) demonstrated good control of *Fusarium* species and *M. nivale* *in vitro* with a range of fungicides. Most isolates of *M. nivale* tested by these workers showed approximately 80-90% inhibition of growth in response to the dicarboximide fungicide iprodione at a concentration of 5mg/l active ingredient, and 80% of *M. nivale* isolates were completely sensitive to the imidazole fungicide prochloraz at 0.05mg/l a.i. Limited evidence for the effective control of *Fusarium* by fungicides in the field is given by Lipatoff & Lartaud (1983) who demonstrated that fenpropimorph in combination with carbendazim gave control of *Fusarium* foot rot on both wheat and barley, and by Formagoni & Vangoni (1984), who showed that foliar fungicide spray applications at growth stage 31 (first node detectable), of a formulation of thiophanate-methyl and maneb gave a 220kg/ha yield increase over untreated control plots. It is particularly difficult to measure the efficacy of fungicides against individual

Fusarium species and *M.nivale* in the field, as disease symptoms may be caused by one or several species, and the relationship between disease symptoms and their effect on yield is not understood. There is also a problem in that individual species may or may not be isolated using conventional techniques. There may also be an interaction between *Fusarium* species and the more important and naturally occurring stem base pathogen *Pseudocercospora herpotrichoides*.

The reasons for the disparity between the effectiveness of fungicides in laboratory sensitivity tests and in the field are not clear, and potential contributory factors are manifold. In the case of protectant fungicides, Keil *et al.* (1952) reported that given equal ED50 values, organic fungicides performed in the field in the order of their resistance to rainfall, but noted that there was considerable variability in field performance of individual fungicides. Rich *et al.* (1953) proposed that laboratory data, such as estimation of ED50, dosage response curves and the rate of chemical degradation were useful parameters with which to choose useful fungicides for field use, but suggested that a weighting should be given to each factor dependent on its interactions with the other factors.

Systemic fungicides with eradicant as well as protective properties were introduced in the early 1970's. Benzimidazoles (benomyl, carbendazim, thiabendazole) and benzimidazole-generating fungicides (thiophanates) were the first systemic fungicides with a broad antifungal spectrum. These fungicides bind to tubulin, and therefore affect the function of cellular microtubules, causing inhibition of mycelial growth and distortion of germ tubes (Davidse, 1986). Both benomyl and carbendazim were shown to penetrate the cuticle and to provide local systemic and curative action (Edgington, 1977). Whereas the desired attributes of a protectant fungicide are fungitoxicity, resistance to erosion by rainfall and other atmospheric variables, a systemic fungicide must not only move within the plant but also reach and penetrate the fungus to be

effective.

The ergosterol biosynthesis inhibitors (EBIs) are a group of fungicides which exhibit a wide range of systemic properties. Fungi require ergosterol or closely related sterols for proper function of their cell membranes. These sterols are incorporated into the membrane lipid bilayer, and provide strength and flexibility. Sterol biosynthesis starts from acetic acid and involves about 30 steps. There are two sub-groups of the EBIs; morpholines, which inhibit reduction or rearrangement of C = C double bonds in the ergosterol biosynthesis pathway, and are commonly used to control powdery mildew (Brown & Evans, 1992), and de-methylation inhibitors (DMIs). DMI fungicides selectively inhibit a single step in the sterol biosynthesis pathway, the removal of a single methyl group at the 14 position of the steroid nucleus. The demethylase enzyme active site is bound by the fungicide, and the sequence of reactions which would normally remove the methyl group cannot begin. With the inhibitor bound, neither oxygen nor sterol can enter, and 14-methyl sterols accumulate in the cell. Fungitoxicity of demethylation inhibitors has been attributed to depletion of ergosterol and to accumulation of sterol precursors in the fungal membranes. The abnormal sterol content causes membrane hyperfluidity, leading to changes in membrane permeability and activity of membrane bound enzymes (Kato, 1986). Two important fungicides used for the control of wheat stem base disease in the UK are firstly Sportak (40 % w.v. prochloraz, AgrEvo UK Ltd, Chesterford Park, Essex) an imidazole EBI fungicide widely used for controlling eyespot disease of cereals caused by *P.herpotrichoides*, and also other stem base pathogens, and secondly Genie (40% w.v. flusilazole, E.I. Du Pont de Nemours, Wilmington, Delaware) a DMI triazole fungicide developed to combat a wide range of fungal diseases in various crops (Fort & Moberg, 1984). In field trials in France (Fort & Moberg, 1984) and England (King & Griffin, 1985), flusilazole was found to be as effective as prochloraz against eyespot, but in further

work (Austin, 1986) showed less activity than prochloraz. It is possible that flusilazole is less effective against the R-type of eyespot than prochloraz (Cavelier *et al.*, 1992). There is no record of the relative performance of these fungicides in controlling *Fusarium* species and *M.nivale*.

The effect of triazole fungicides on fungal conidia has been investigated. Henry, (1990) examined the effect of flusilazole on the germination of conidia of *Ustilago maydis*, and found that the treated spores were branched and swollen. Pontzen & Scheinpflug (1989) found that the synthesis of sterols in germinating spores of *Botrytis cinerea*, *Venturia inaequalis* and *Puccinia graminis* f. sp. *tritici* was strongly inhibited by triazole fungicides, and Waterfield & Sisler (1989) showed that the germination of sclerotia of *Sclerotium rolfsii* could be inhibited by the presence of the triazole fungicide propiconazole. Al-Ayoubi & Shephard (1990) compared the action of the triazole fungicide flusilazole with two other triazoles flutriafol and propiconazole and with the morpholine fungicide fenpropimorph and found that the vapour activity of flusilazole was much higher than the other fungicides tested, and could therefore have a greater effect in the reduction of viable conidia on the leaf surface before penetration.

Despite the lack of control of pathogens by fungicides in the field, it is clear that fungicides are able to inhibit conidial germination and growth of mycelium of foot rot pathogens *in vitro*. Inconsistent field performance may be associated with the inability of the fungicide to reach the target pathogen in the field, or effects on the efficacy of the fungicide caused by environmental factors. For example, the variable field performance of prochloraz against *P.herpotrichoides* has been attributed to poor systemic activity, and the need for rainfall to redistribute the fungicide across leaf and stem surfaces (Cooke *et al.*, 1993).

The timing of fungicide applications is a critical factor for pathogen control, but the epidemiology of *Fusarium* species, which is important when planning fungicide spray

programmes is not well understood. In a study of stem-base infection throughout the growing season, Parry (1990) suggested that fungicides applied to control eyespot disease of cereals (GS31) were premature to the main phase of infection and development of *Fusarium* species, and that much later sprays may fail to reach the sites of *Fusarium* infection. In addition, inconsistent field performances may also be related to fungicide resistance in populations of *Fusarium* species and *M.nivale*. Resistance to the methyl benzimidazole carbamate (MBC) fungicides once used extensively to control foot rot disease of wheat has been widely recognised. Locke *et al.* (1987) found that 92.1 % of isolates of *M.nivale* tested were resistant to the MBC fungicide benomyl, and MBC resistance in *M.nivale* has also been reported in Germany (Hartke & Buchenauer, 1985), Sweden (Olvang, 1984) and Japan (Tanaka *et al.*, 1983). Polley *et al.* (1991) noted that *M.nivale* showed a wide range of sensitivity to prochloraz, flusilazole and tebuconazole *in vitro*, which may indicate the development of insensitivity to this group of DMI fungicides.

This work was carried out in order to address the problems of fungicide efficacy against the stem base disease of wheat caused by a combination of several *Fusarium* species, and *M.nivale*. Due to the possible co-existence of different *Fusarium* species and *M.nivale* within a stem base, or within a single lesion, it was necessary to investigate the effect of the fungicides on each of the individual pathogens. It is possible that some of the pathogens cause more severe disease than others, and that fungicidal inhibition of the less important pathogens may have no effect on disease severity. Alternatively, inhibition of a particular species may confer an advantage to the other species present by reducing competition. In order to clarify this situation, the effect of competition between species needs to be studied.

The effect of the triazole fungicide flusilazole, and the imidazole fungicide prochloraz on conidial germination, germ-tube elongation, inhibition and rate of mycelial growth

of several isolates of *F.culmorum*, *F.avenaceum* and *M.nivale* *in vitro* was examined. In order to examine the relationship between the ability of these fungicides to control the pathogens *in vitro* and *in vivo*, field trials were also undertaken to study the relationship between fungicide concentrations, mixtures, rates of application and timing of application, and disease symptoms, infection and yield of wheat.

1.1 Effect of a triazole and an imidazole fungicide on the conidial germination, germ tube extension and mycelial growth of *Fusarium culmorum*, *Fusarium avenaceum* and *Microdochium nivale* in vitro.

MATERIALS AND METHODS

Germination of conidia of *F.culmorum*, *F.avenaceum* and *M.nivale* in the presence of flusilazole or prochloraz.

Isolates of *F.culmorum*, *F.avenaceum* and *M.nivale*, were obtained from stem bases of wheat (var.Mercia) grown in field plots at Harper Adams, and maintained on PDA at 20°C under a 12 hour near ultra-violet light/ 12 hour dark regime for approximately 10 days, or until sporodochia were produced. Spores were harvested by flooding the PDA plates with sterile distilled water, and scraping the colony surface with a sterile needle. The resulting spore suspension was decanted and strained through 2 layers of muslin to remove hyphal material. The concentration of spores in the solution was adjusted to 4×10^6 spores/ml using an haemocytometer.

Tap Water agar (TWA) was amended with various concentrations of flusilazole or prochloraz to produce concentrations of 0.05, 0.1, 1.0, 10.0, $\mu\text{g/ml}$ a.i.. Unamended TWA was used as a control, and each treatment had 4 replicates. Cellophane squares, 2 cm in diameter, were boiled for 20 minutes in three changes of sterile distilled water to remove sugars, and were then sterilised by autoclaving at 101Kpa for 20 minutes. Two cellophane squares were placed on the surface of each plate of agar, and 100 μl aliquots of the spore suspension were placed on the surface of the cellophane. The plates were incubated at 20°C for 3, 6, 9, 12 and 24 hours. Lactophenol Cotton Blue was then added to the cellophane squares, and 400 spores per treatment were assessed for germination. Germination was considered to have occurred when germ tube length was equal to the width of the spore. After the 12 hour incubation period, the lengths of 30

germ tubes were measured for each treatment using an eyepiece graticule at a magnification of x40, the mean length was calculated, and the measurement calibrated to μm using a microscope stage graticule.

Mycelial growth of *F.culmorum*, *F.avenaceum* and *M.nivale* in the presence of prochloraz or flusilazole.

Five isolates each of *F.culmorum*, *F.avenaceum* and *M.nivale* were obtained from stem-bases of wheat taken from field plots at Harper Adams Agricultural College. Isolates were grown on Potato Sucrose Agar (PSA) at 20°C, and maintained at 5°C. Fungicide supplemented media were made by autoclaving PDA at 101Kpa for 20 minutes, and allowing the solution to cool to 50°C before adding various concentrations of either of the fungicides flusilazole (E.I Du Pont de Nemours, 40% w/v active ingredient) or prochloraz (AgrEvo UK Ltd, 40% w/v a.i.). The PDA was supplemented with fungicide solutions, to produce concentrations of 0.1, 1.0, 2.0, 10.0, and 50.0 $\mu\text{g/ml}$ a.i. fungicide, and poured into 9.5cm diameter Petri dishes at a rate of 20ml/plate. Non-amended plates were used as controls, and four replicates of each treatment were used. The plates were inoculated with 5mm plugs of mycelium taken from the edge of an actively growing colony of *F.culmorum*, *F.avenaceum* or *M.nivale*. In the first test, one isolate of each of the 3 species was tested against the range of fungicides in order to determine a typical dose response curve. Subsequently, five isolates of each species were tested, and the average percentage inhibition for the species calculated. Plates were incubated at 20°C, and the colony diameter of each of the isolates measured at regular intervals until the colony diameter of the control plates for each particular species had reached approximately 90mm. The concentration of fungicide required to inhibit the growth of each of the three species by 50% was calculated by probit analysis of the four replicates. A regression line was fitted to the data, and the concentration at

50% inhibition estimated to provide an EC50 value .

RESULTS

Conidia of *M.nivale* began to germinate after 3 hours incubation. The presence of flusilazole had no effect on the final percentage germination at concentrations of 0.05, 0.1 and 1.0 $\mu\text{g/ml}$ a.i., but at 10 and 50 $\mu\text{g/ml}$ a.i. flusilazole, conidial germination was almost totally inhibited (Fig 1.1.1). Prochloraz was more effective than flusilazole in slowing down germination of conidia of *M.nivale* at all concentrations; at 0.1 $\mu\text{g/ml}$ a.i. percentage germination after 24 hours was reduced slightly (Fig 1.1.2), and at 1, 10 and 50 $\mu\text{g/ml}$ a.i. germination was greatly inhibited. The average germ tube length of *M.nivale* conidia measured after 9 hours incubation was reduced by increasing concentrations of flusilazole (Fig 1.1.3), and totally inhibited at 10 and 50 $\mu\text{g/ml}$. Prochloraz (Fig 1.1.3) reduced the length of the germ tubes to a slightly greater extent, but total inhibition did not occur at the highest concentrations.

The mycelial growth rate of *M.nivale* over 9 days was reduced by each increase in concentration of flusilazole (Fig 1.1.4). The colonies on the unamended control plates reached 90mm growth after 7 days incubation, with an average growth rate of 9.9mm/day. Successive increases in flusilazole, reduced the average growth rate of *M.nivale*, with concentrations of 1.0 $\mu\text{g/ml}$ and 10.0 $\mu\text{g/ml}$ a.i. reducing the rate to 3.43mm/day and 3.26mm/day respectively, and further concentrations totally inhibiting growth of the fungus. Prochloraz was not as initially effective as flusilazole in reducing growth rate of *M.nivale* (Fig 1.1.5), with a reduction from 9.9mm/day in the control, to 5.76mm/day at a concentration of 1 $\mu\text{g/ml}$ a.i.. However, growth was completely inhibited at 10 $\mu\text{g/ml}$ prochloraz. *M.nivale* showed a gradual dose response to increasing concentration of prochloraz (Fig 1.1.6), and growth was totally inhibited at 10 $\mu\text{g/ml}$ a.i. The presence of flusilazole produced a similar result, with a gradual

increase in growth inhibition related to fungicide concentration. The concentrations of flusilazole and prochloraz required to inhibit the growth of *M.nivale* by 50% (EC50), calculated from the dose response curves (Fig 1.1.6) are illustrated in Table 1.1. The EC50 values for *M.nivale* were 1.035 $\mu\text{g/ml}$ for flusilazole, and 0.89 $\mu\text{g/ml}$ for prochloraz.

Conidia of *F.culmorum* began to germinate after between 6 and 9 hours incubation. After 9 hours incubation with flusilazole a clear concentration dependant effect on rate of germination was recorded (Fig 1.1.7). However, after 12 hours, no effect of concentration was noted, and all conidia had germinated. Prochloraz produced a similar effect on germination after 9 hours (Fig 1.1.8) with percentage germination being dependant on fungicide concentration, but in this case the trend continued at 12 and 24 hours incubation, particularly for concentrations greater than 1.0 $\mu\text{g/ml}$ a.i. The average germ tube length produced by *F.culmorum* conidia was greatly affected by flusilazole after 9 hours (Fig 1.1.9). Although 100% of conidia germinated after 24 hours, the length of the germ-tubes which they had produced was dependant on flusilazole concentration, with a reduction from 105 μm length in the control to 32 μm at a concentration of 50 $\mu\text{g/ml}$. Prochloraz (Fig 1.1.9) produced a very similar, but slightly more inhibitory effect on germ tube elongation than flusilazole.

The average mycelial growth rate of *F.culmorum* was reduced by increasing flusilazole concentration from the control level of 10.91mm/day to 5.33mm/day at 1.0 $\mu\text{g/ml}$ a.i. and total inhibition at 10 and 50 $\mu\text{g/ml}$ a.i. (Fig 1.1.10). Prochloraz (Fig 1.1.11) had a more inhibitory effect than flusilazole at lower concentrations, with an immediate reduction from 10.91mm/day in the controls to 3.66mm/day at 0.1 $\mu\text{g/ml}$ a.i., and total inhibition at 1 $\mu\text{g/ml}$ a.i.. Flusilazole (Fig 1.1.12) had a similar effect on *F.culmorum*, as *M.nivale* in terms of mycelial growth inhibition with an EC50 value of 0.89 $\mu\text{g/ml}$. Prochloraz was much more inhibitory at low concentrations (Fig 1.1.12) and had an

EC50 value of less than 0.1 μ g/ml a.i.

Conidia of *F.avenaceum* germinated after 6 hours incubation (Fig 1.1.13). The rate of germination was slightly affected by concentration of flusilazole at 6 and 9 hours, but no effect was observed after 24 hours, when all treatments had reached 100% germination. Prochloraz had a much greater effect on conidial germination of *F.avenaceum* (Fig 1.1.14), than flusilazole at all concentrations, with inhibition of germination occurring after 24 hours. Germ-tube length of *F.avenaceum* was decreased by increasing flusilazole concentration, but to a lesser extent than *F.culmorum* (Fig 1.1.15) and prochloraz produced a very similar effect. The mycelial growth rate of *F.avenaceum* was reduced by increasing concentration of flusilazole (Fig 1.1.16) from 9.9mm/day in the control to 1.1mm/day in the 50 μ g/ml treatment, each increase in fungicide produced a decrease in growth rate, apart for an anomaly in the results of the 1 μ g/ml treatment after 7 days incubation. Prochloraz was more effective than flusilazole in reducing growth rate of *F.avenaceum* at low concentrations, with an immediate reduction from 9.9mm/day in the control, to 4.14mm/day at 0.1 μ g/ml, and complete inhibition at higher concentrations (Fig 1.1.17). The EC50 of prochloraz calculated from the dose response curves (Fig 1.1.18) was less than 0.1 μ g/ml, and that of flusilazole 8.16 μ g/ml (Table 1.1).

	Species		
Fungicide	<i>M.nivale</i>	<i>F.culmorum</i>	<i>F.avenaceum</i>
Flusilazole	1.035	0.89	8.16
Prochloraz	0.89	< 0.1	< 0.1

Table 1.1.1 Concentration of the fungicides flusilazole and prochloraz (E.C.50 values ($\mu\text{g/ml a.i.}$)) estimated to inhibit by fifty percent the mycelial growth of the species *Fusarium culmorum*, *F.avenaceum* and *Microdochium nivale* *in vitro*.

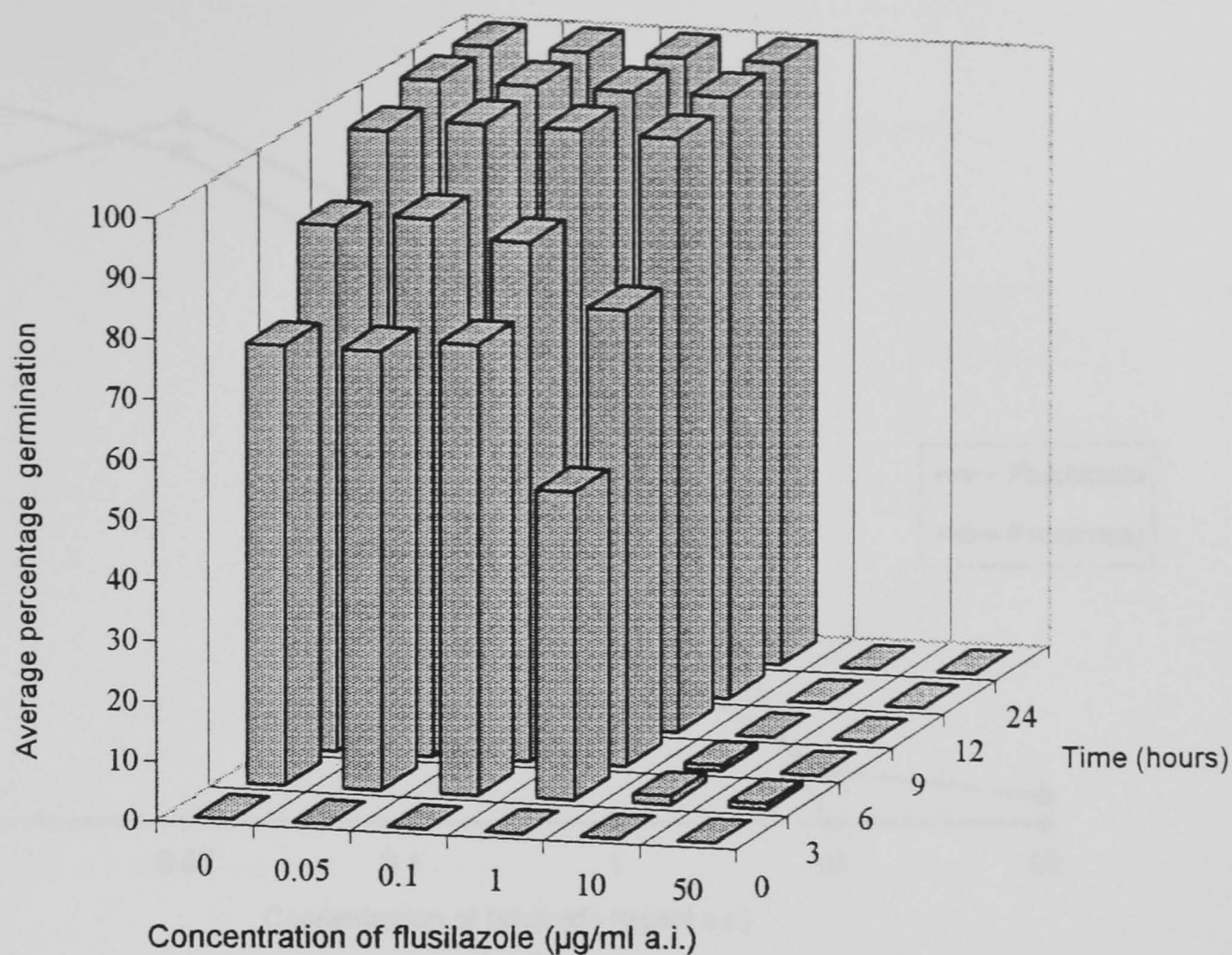


Fig 1.1.1 Average percentage germination of conidia of *M.nivale* at 0, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i. of flusilazole after 0, 3, 6, 9, 12, and 24 hours incubation. For SEM values see appendix 1.1.1.

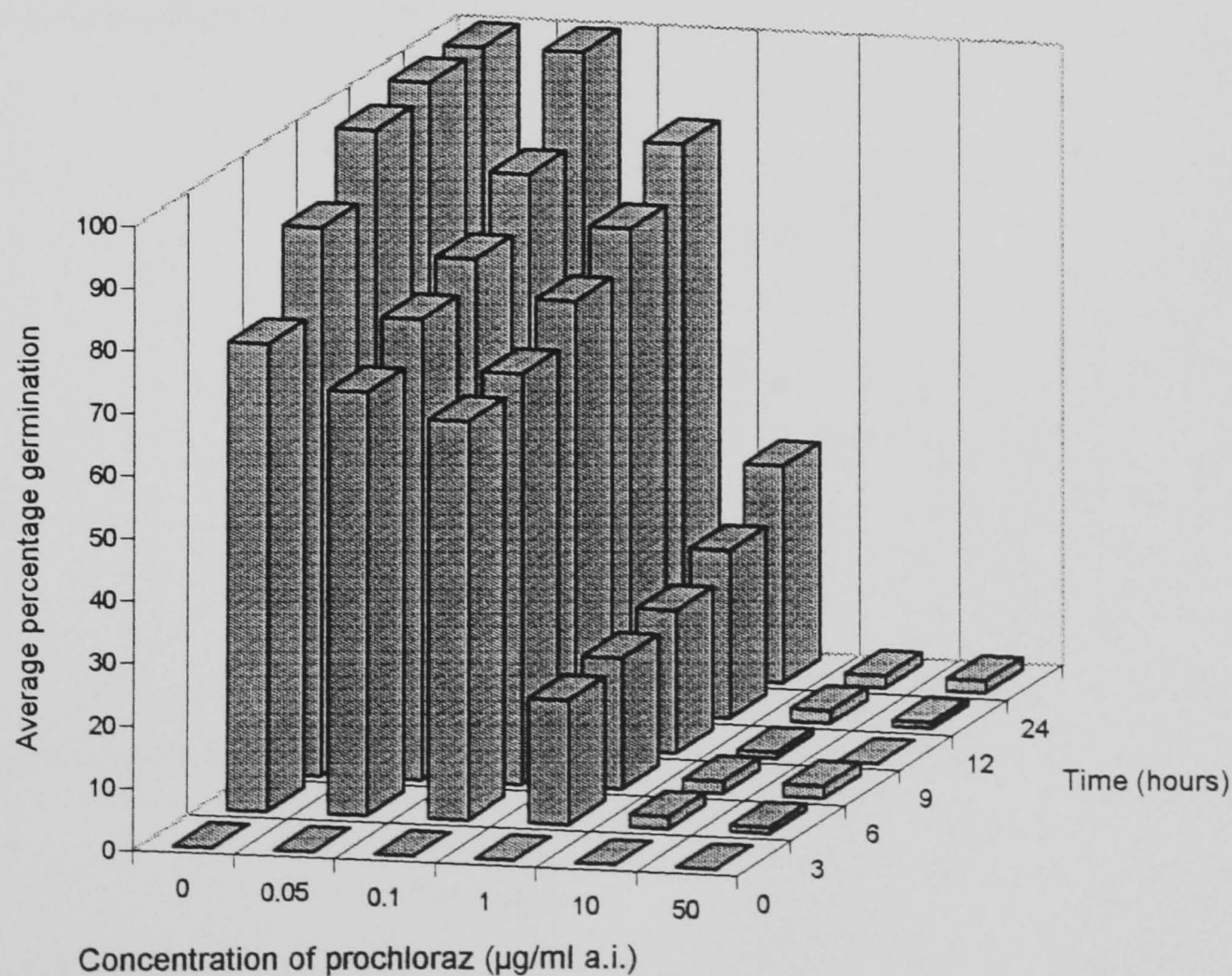


Fig. 1.1.2 Average percentage germination of conidia of *M.nivale* at 0, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i. of prochloraz after 0, 3, 6, 9, 12 and 24 hours incubation. For SEM values see appendix 1.1.2.

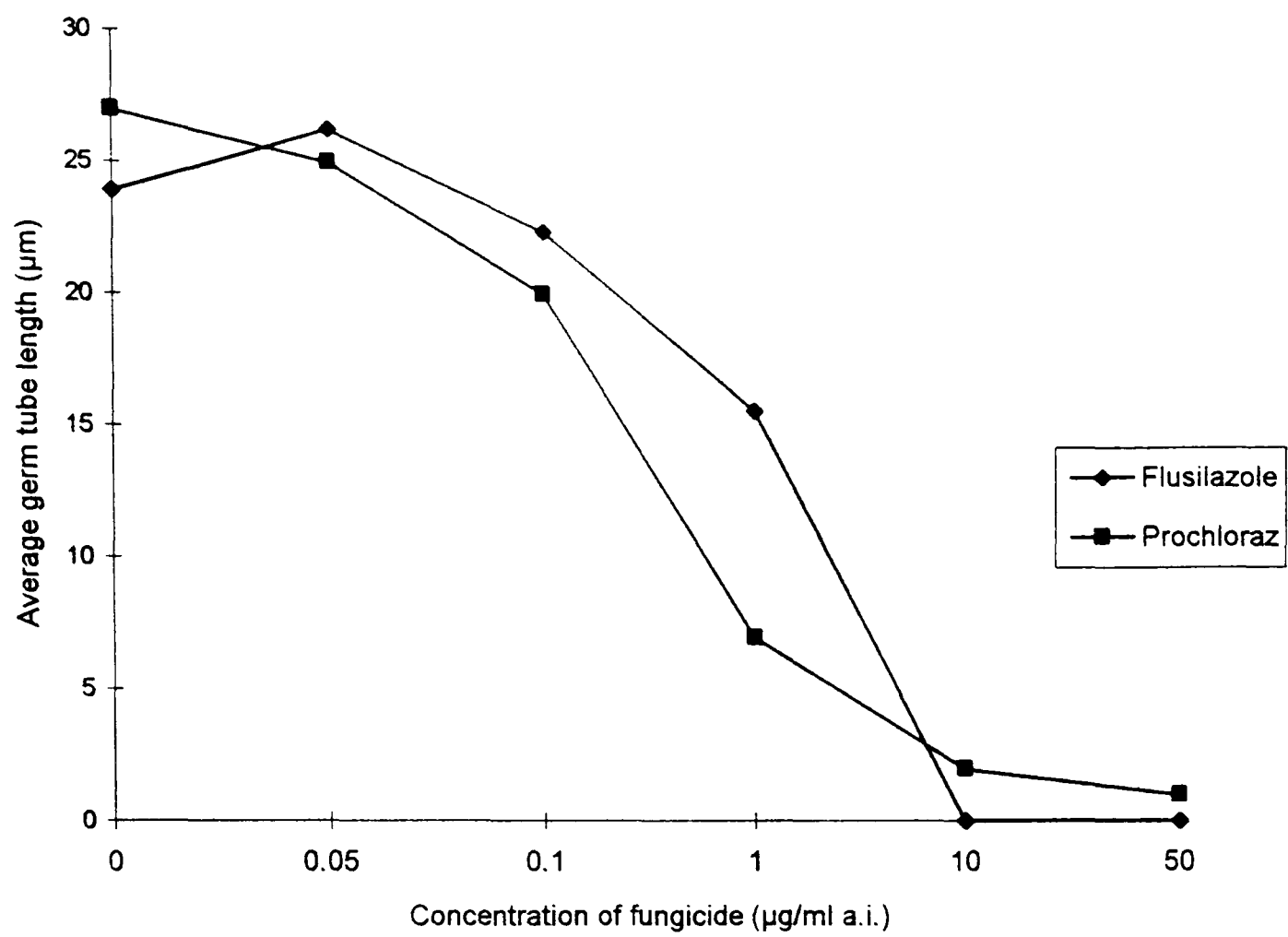


Fig. 1.1.3 The effect of varying concentration (0, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i. of flusilazole or prochloraz on the average germ tube length (µm) of five isolates of *M. nivale* after 9 hours incubation. For SEM values see appendix 1.1.3.

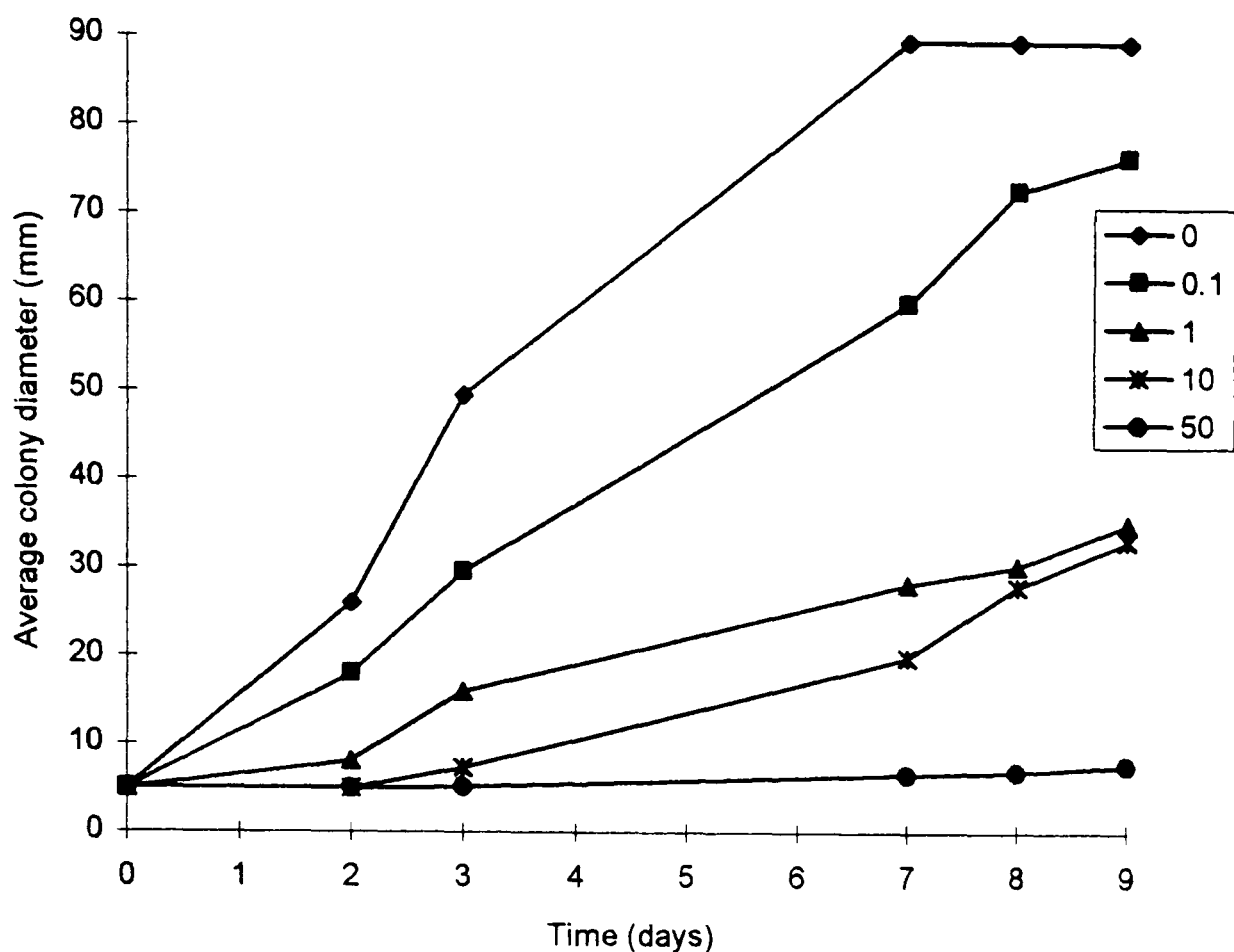


Fig.1.1.4 Effect of varying concentration (0, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i.) of flusilazole on the average colony diameter of five isolates of *M. nivale* at six dates. For SEM values see appendix 1.1.4

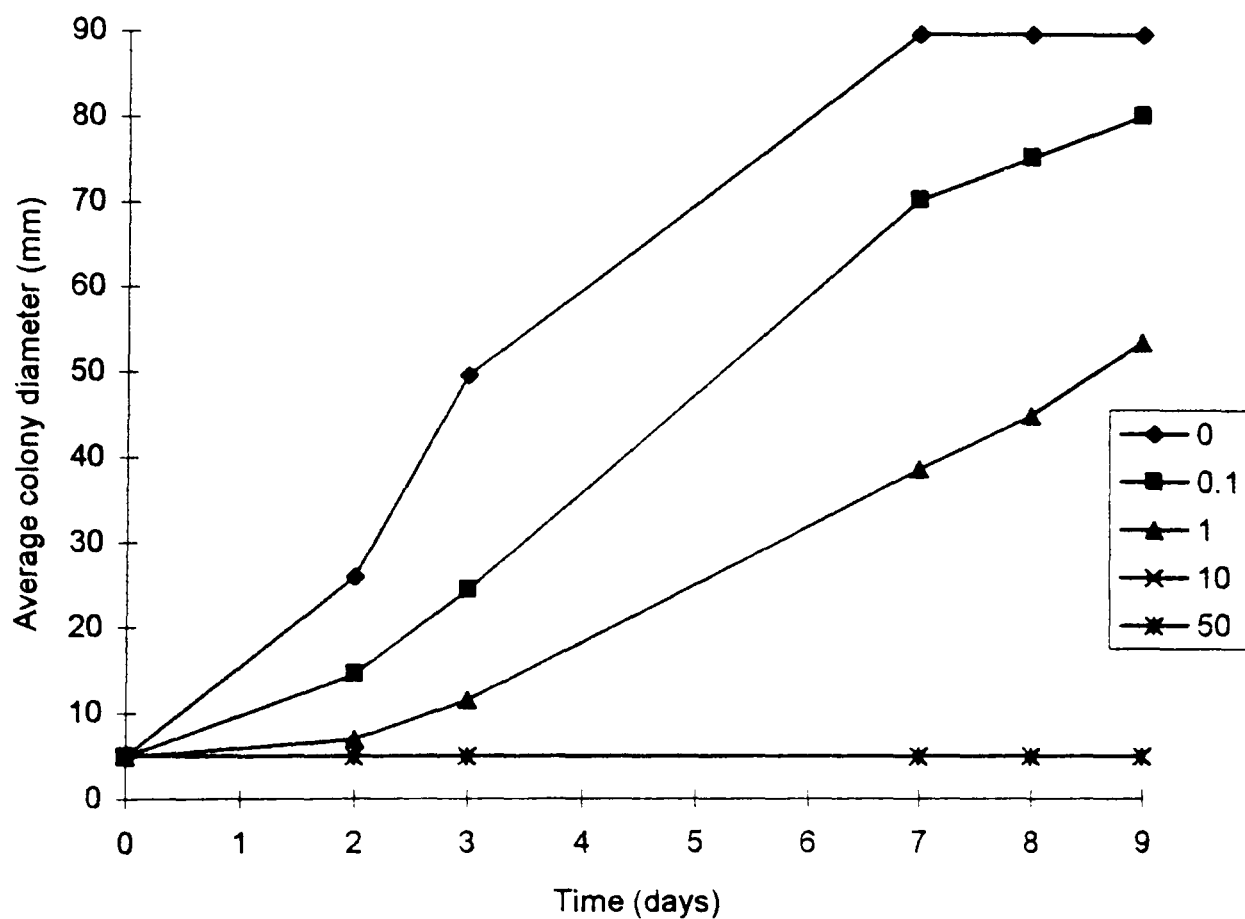


Fig. 1.1.5 Effect of varying concentration (0, 0.1, 1.0, 10.0 and 50.0 0g/ml a.i.) of prochloraz on the average colony diameter (mm) of five isolates of *M. nivale* at six dates. For SEM values see appendix 1.1.5.

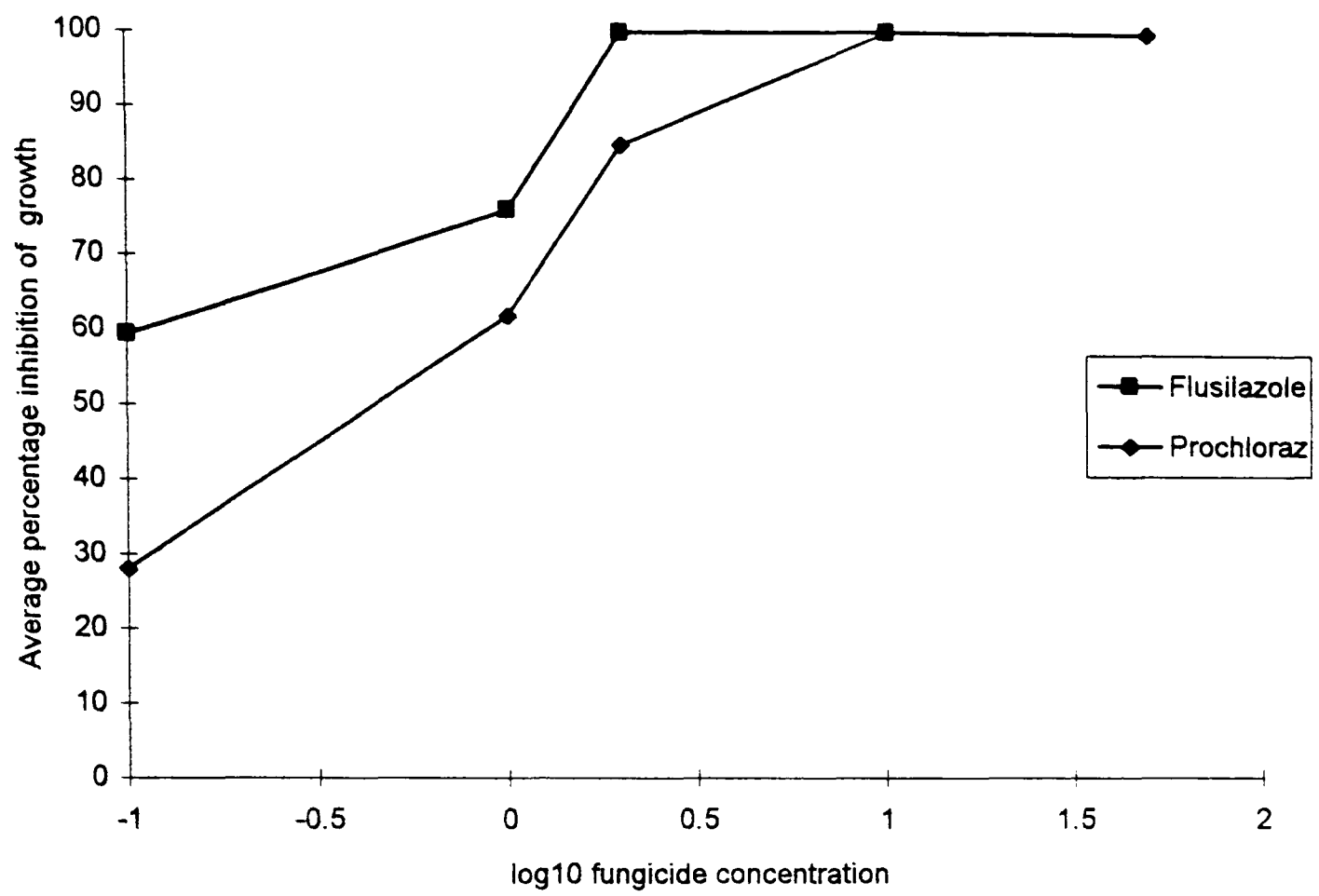


Fig. 1.1.6 The effect of varying concentration (0, 0.05, 0.1, 1.0, 10.0 and 50.0 $\mu\text{g/ml}$ a.i.) of flusilazole and prochloraz on the final average percentage inhibition of mycelial growth of five isolates of *M.nivale* . For SEM values see appendix 1.1.6

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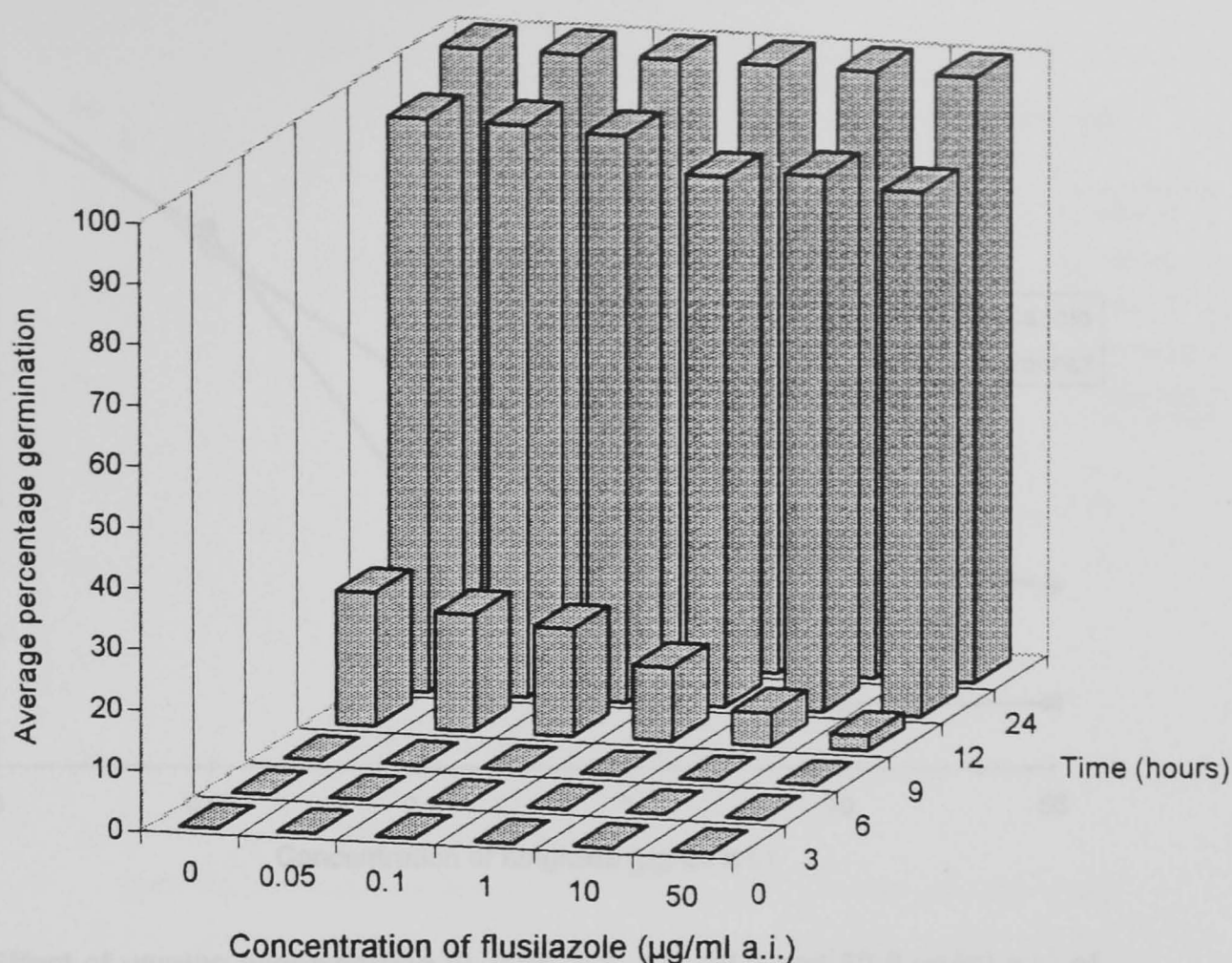


Fig 1.1.7 Average percentage germination of conidia of *F. culmorum* at 0, 0.05, 0.1, 1.0, 10.0, and 50.0 µg/ml a.i. of flusilazole after 0, 3, 6, 9, 12 and 24 hours. For SEM values see appendix 1.1.7

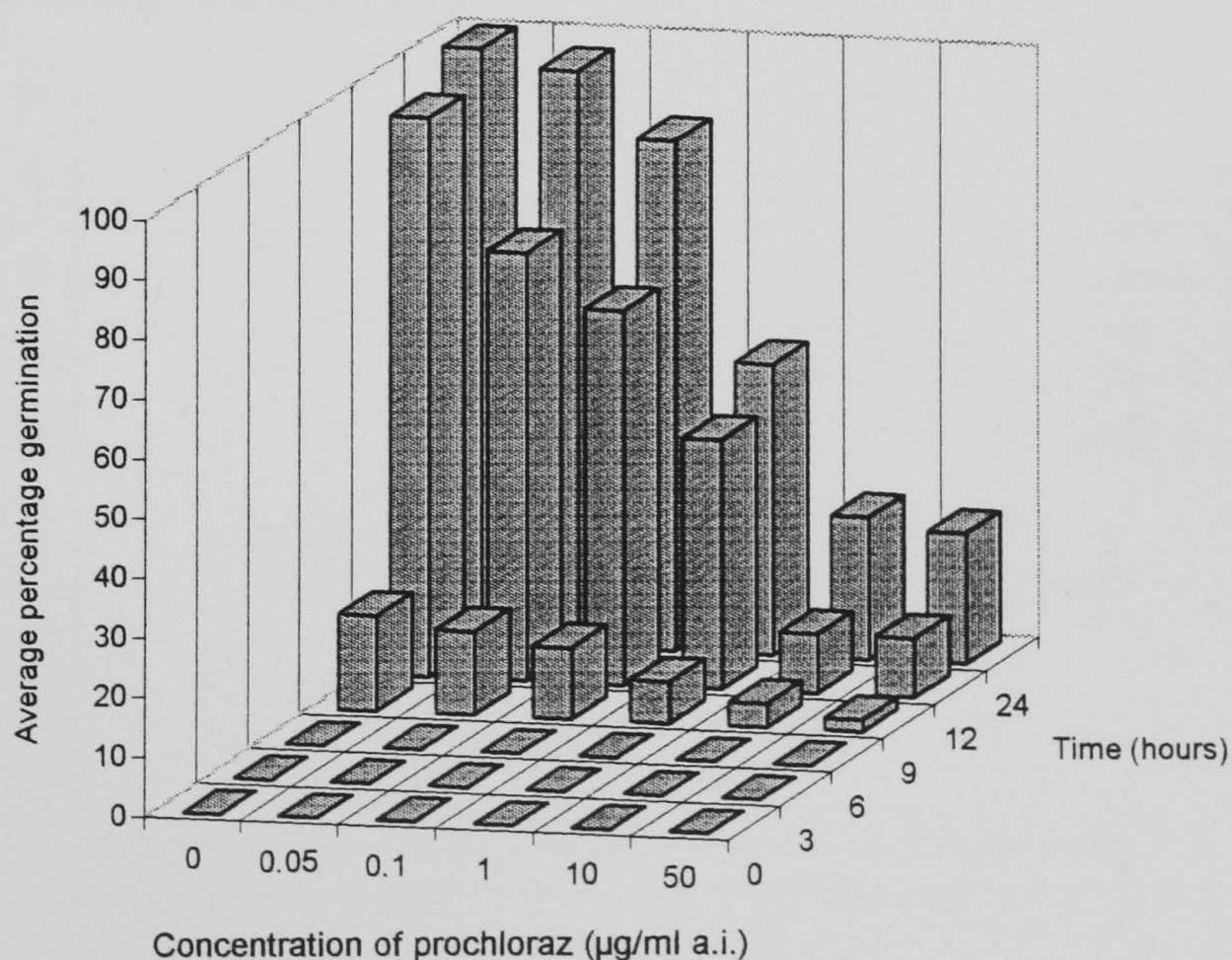


Fig. 1.1.8 Average percentage germination of conidia of *F. culmorum* at 0, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i. of prochloraz after 0, 3, 6, 9, 12 and 24 hours. For SEM values see appendix 1.1.8.

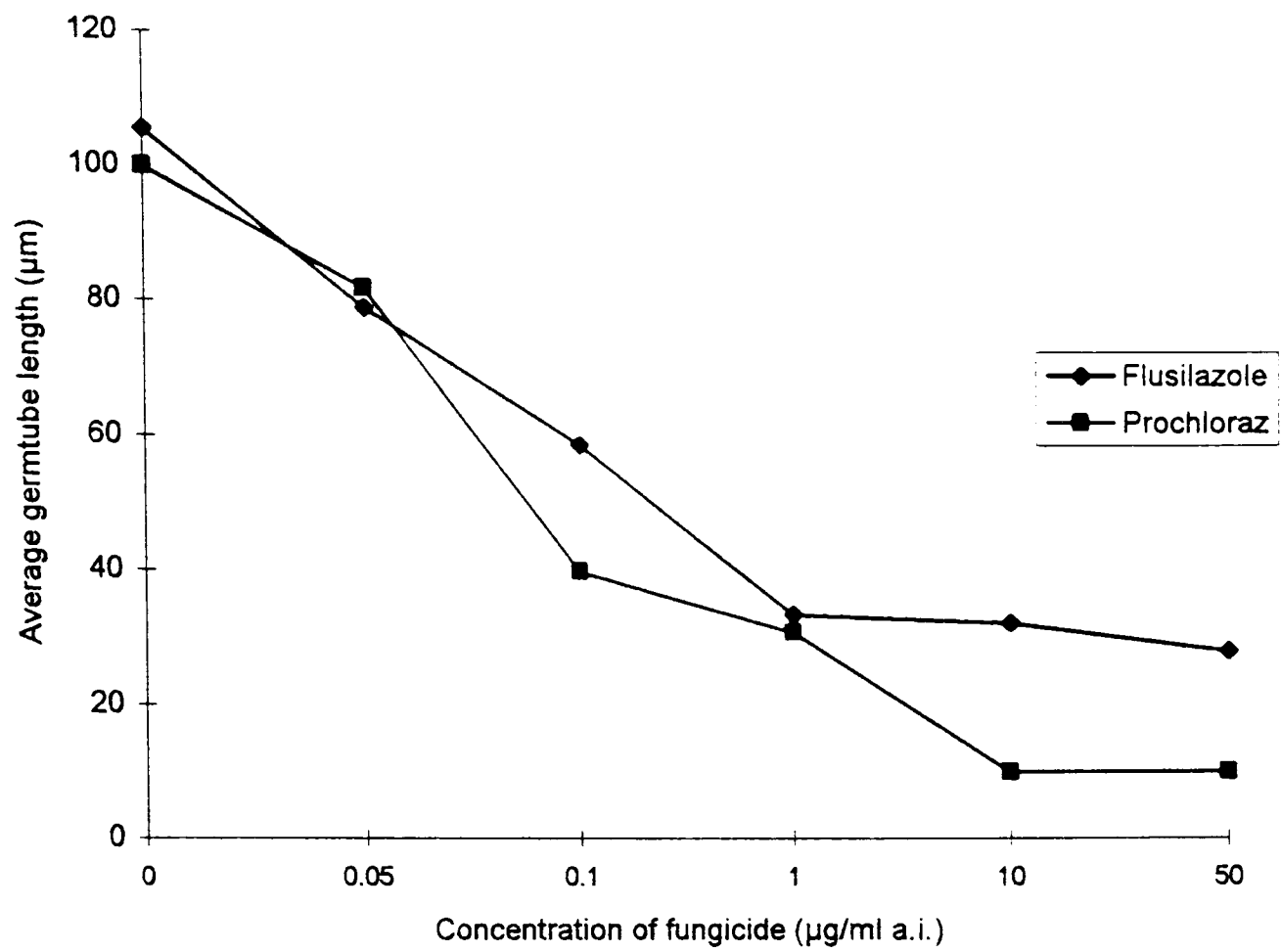


Fig. 1.1.9 Effect of varying concentration (0, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i.) of flusilazole and prochloraz on the average germtube length (µm) of five isolates of *F. culmorum*. For SEM values see appendix 1.1.9

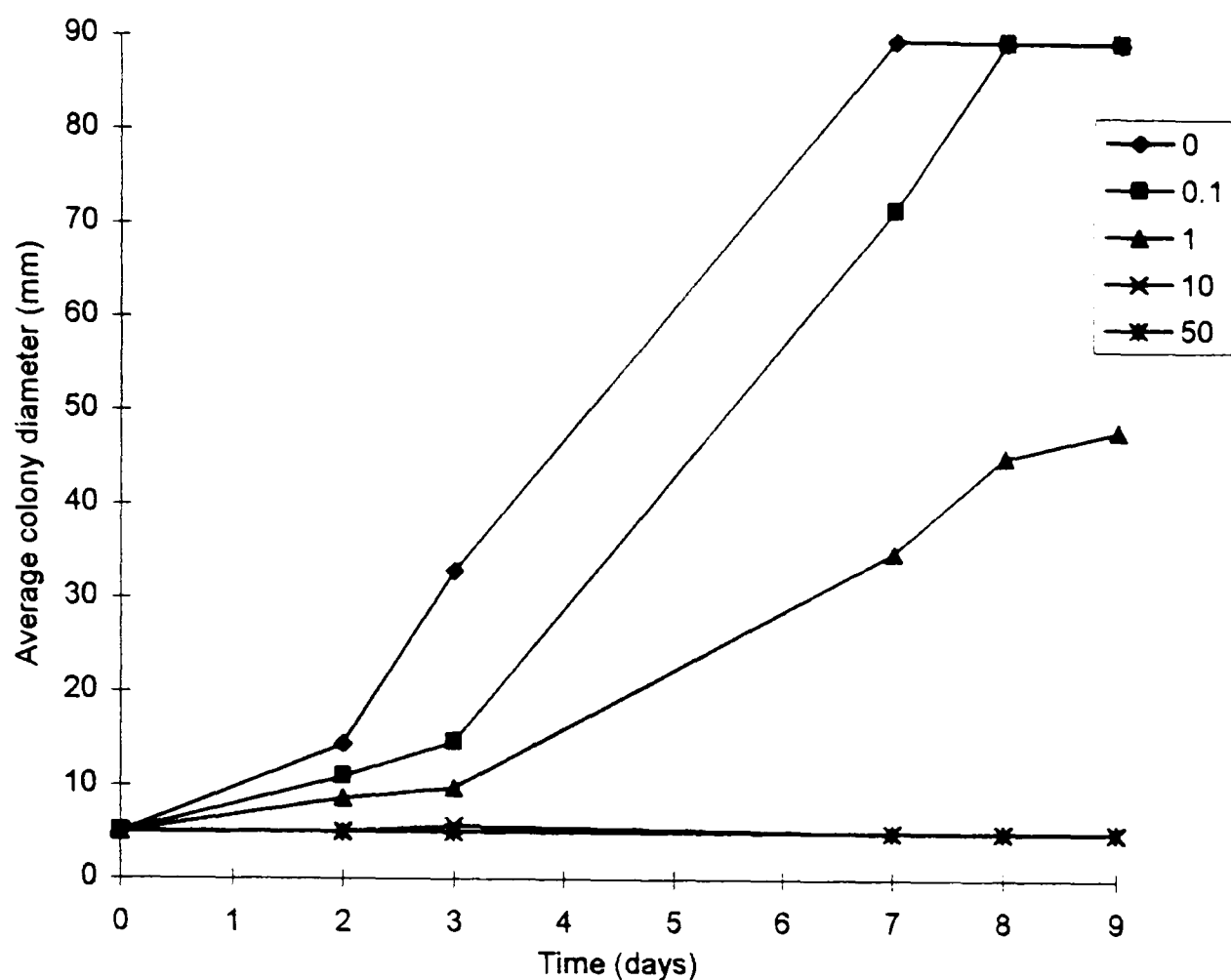


Fig. 1.1.10 Effect of varying concentration (0, 0.1, 1.0, 10.0 and 50.0 $\mu\text{g/ml}$ a.i.) of flusilazole on the average colony diameter (mm) of five isolates of *F. culmorum* at six dates. For SEM values see appendix 1.1.10

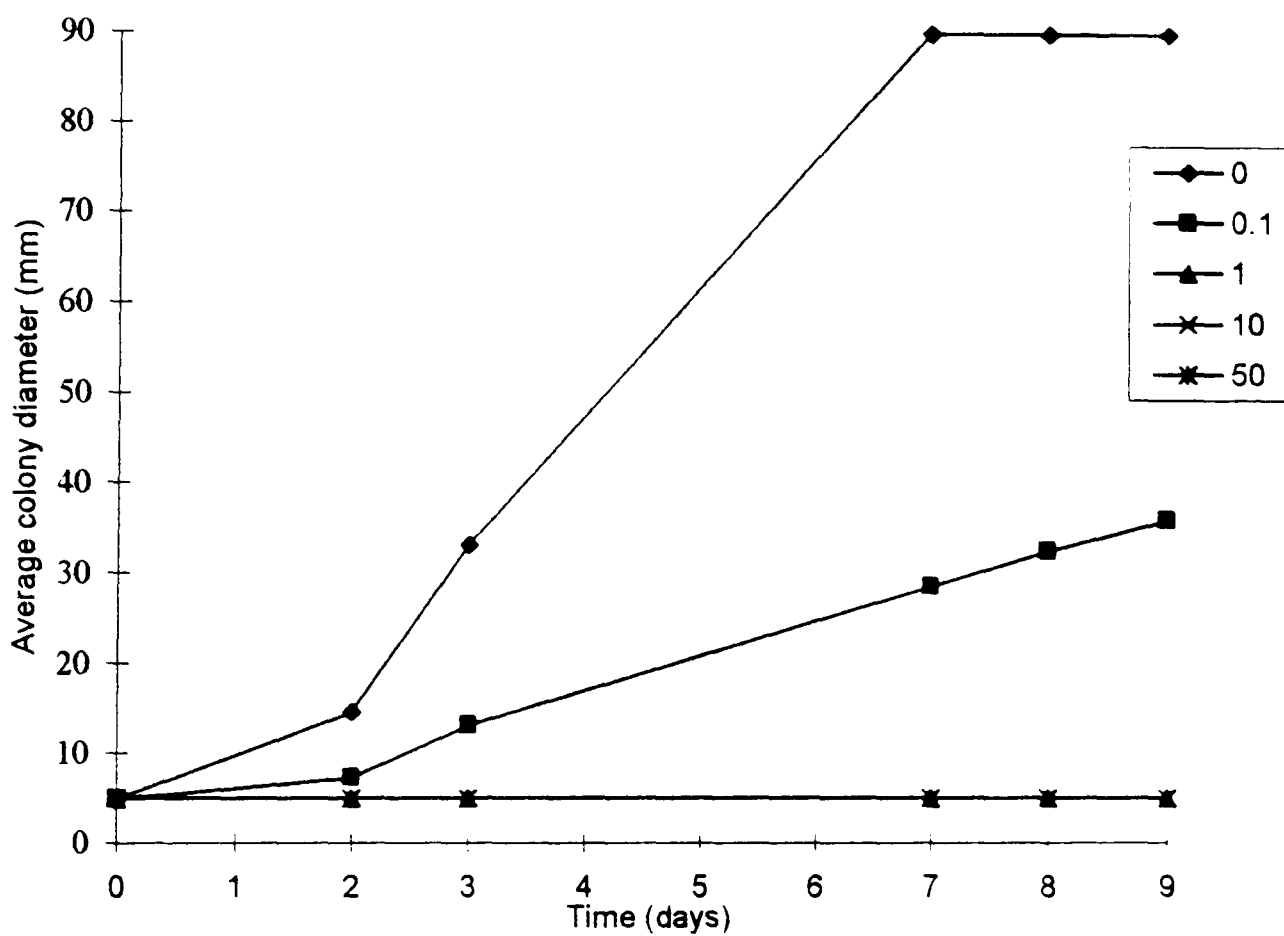


Fig.1.1.11 Effect of varying concentration (0, 0.1, 1.0, 10.0 and 50.0 $\mu\text{g/ml}$ a.i.) of prochloraz on the average colony diameter (mm) of five isolates of *F. culmorum* at six dates. For SEM values see appendix 1.1.11.

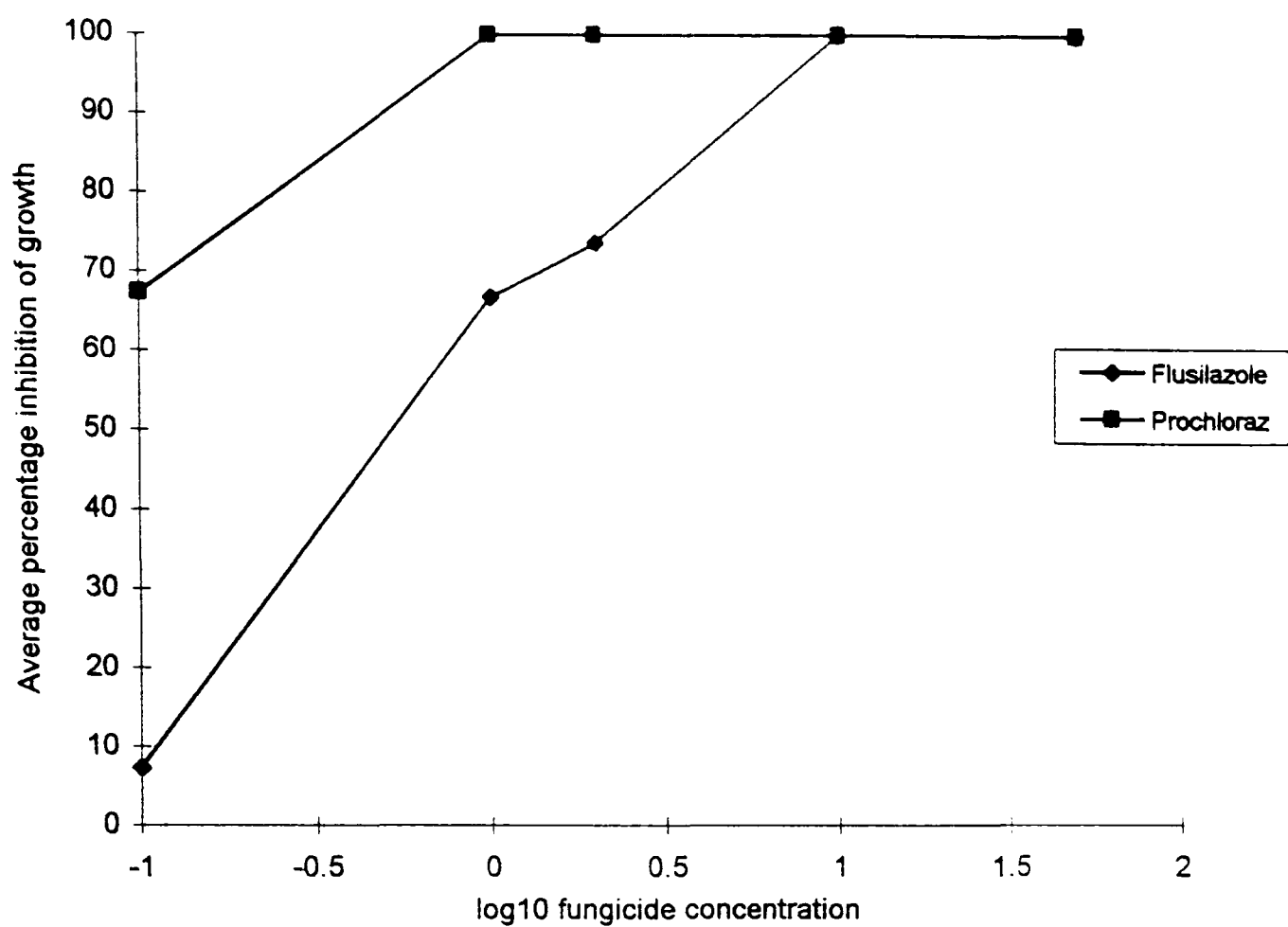


Fig. 1.1.12 Effect of varying concentration (0, 0.1, 1.0, 2.0, 10.0 and 50.0 $\mu\text{g/ml}$ a.i.) of flusilazole and prochloraz on the average percentage inhibition of mycelial growth of five isolates of *F.culmorum*. For SEM values see appendix 1.1.12

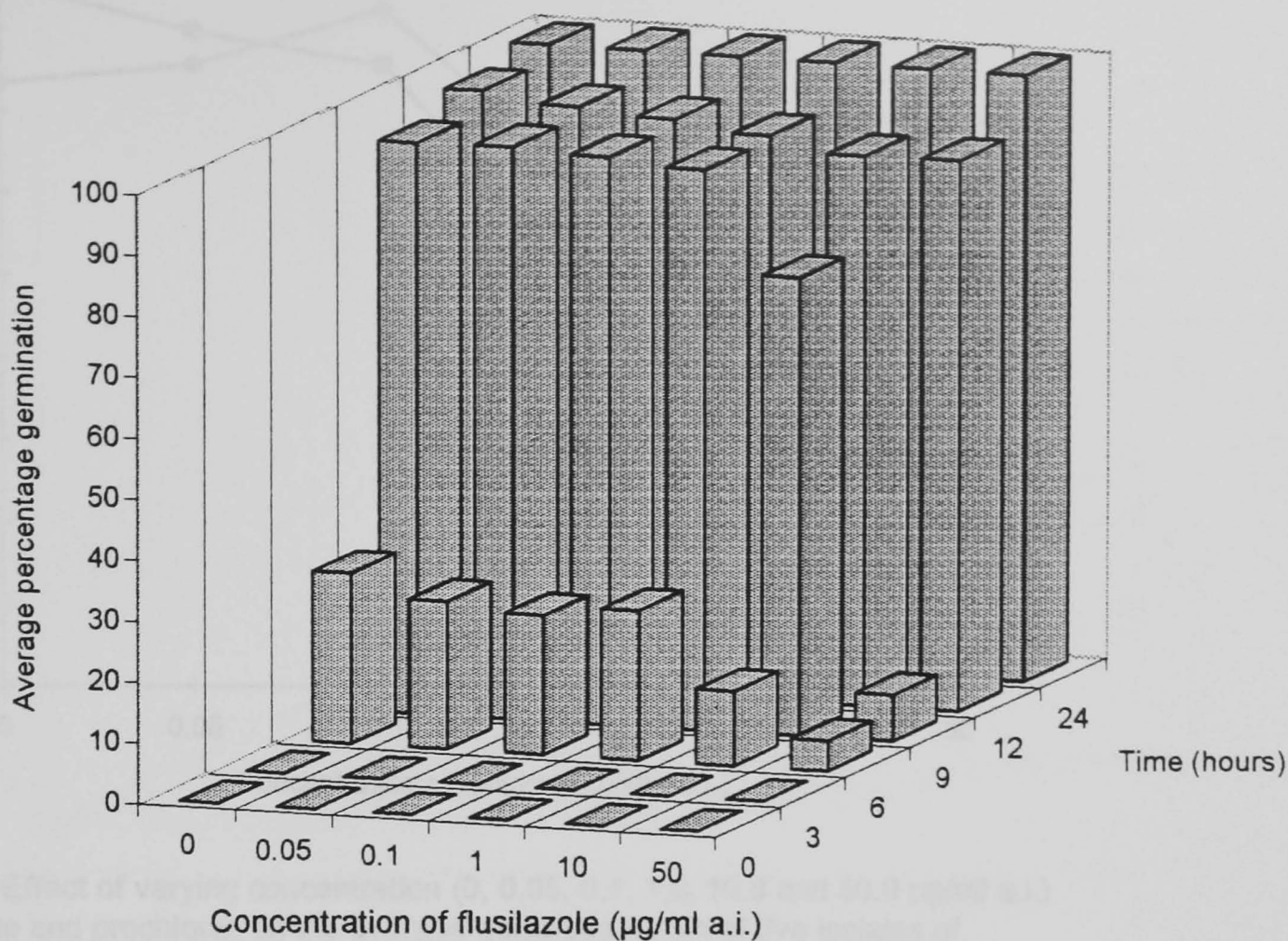


Fig. 1.1.13 Average percentage germination of conidia of *F.avenaceum* at 0, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i. of flusilazole after 0, 3, 6, 9, 12 and 24 hours incubation. For SEM values see appendix 1.1.13.

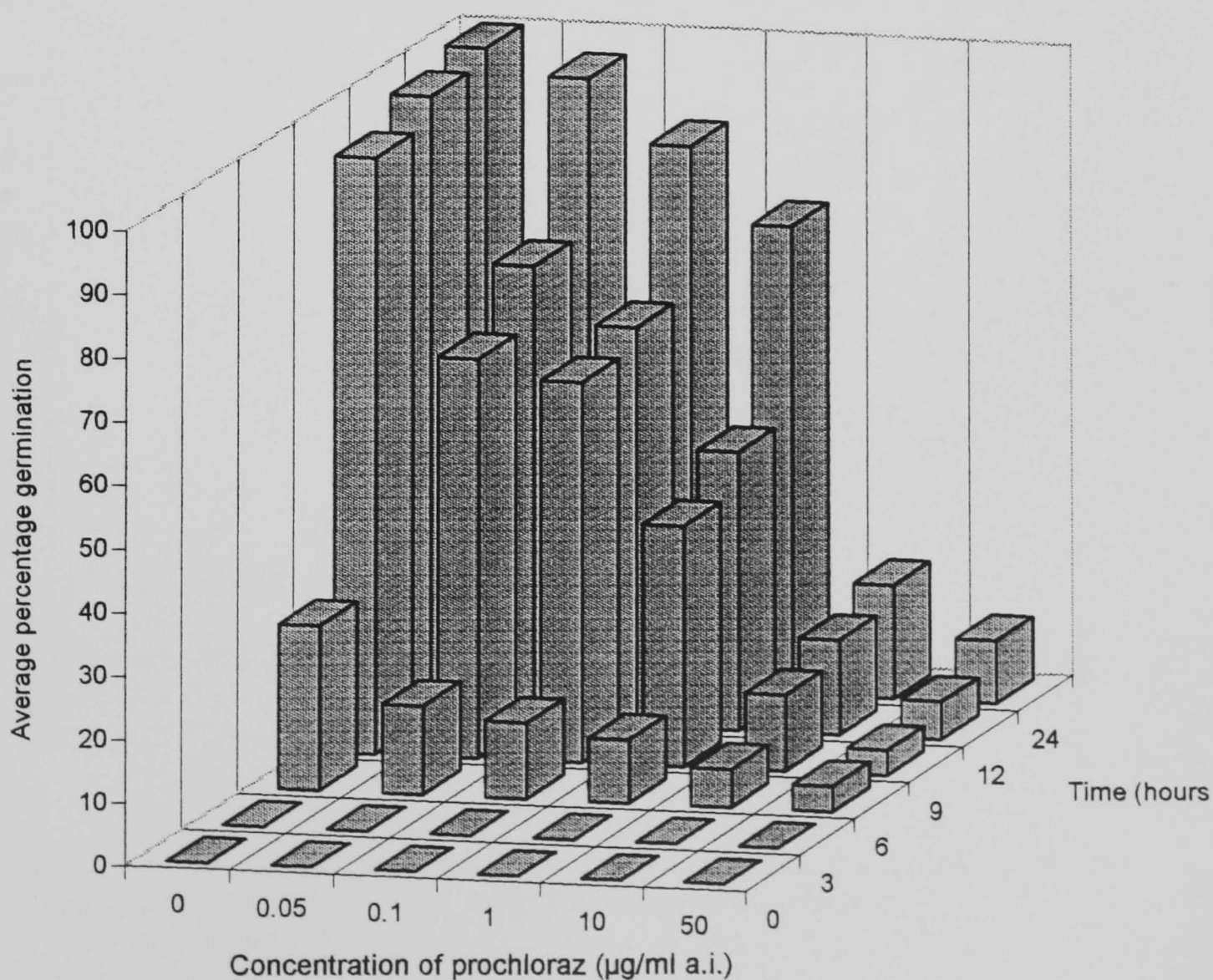


Fig. 1.1.14 Average percentage germination of conidia of *F.avenaceum* at 0, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i. prochloraz after 0, 3, 6, 9, 12 and 24 hours incubation. For SEM values see appendix 1.1.14.

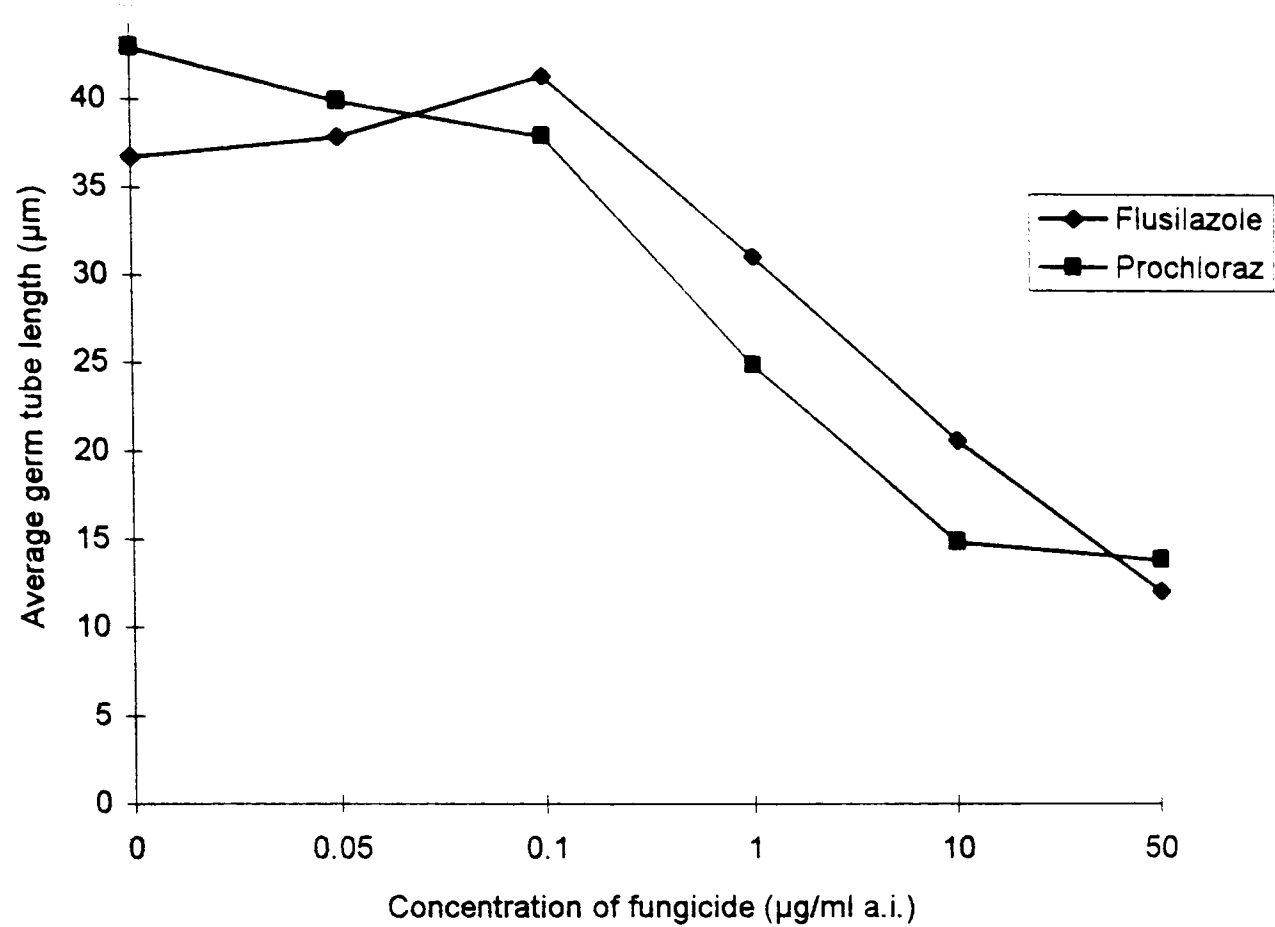


Fig. 1.1.15 Effect of varying concentration (0, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/ml a.i.) of flusilazole and prochloraz on the average germtube length of five isolates of *F.avenaceum* .For SEM values see appendix 1.1.15.

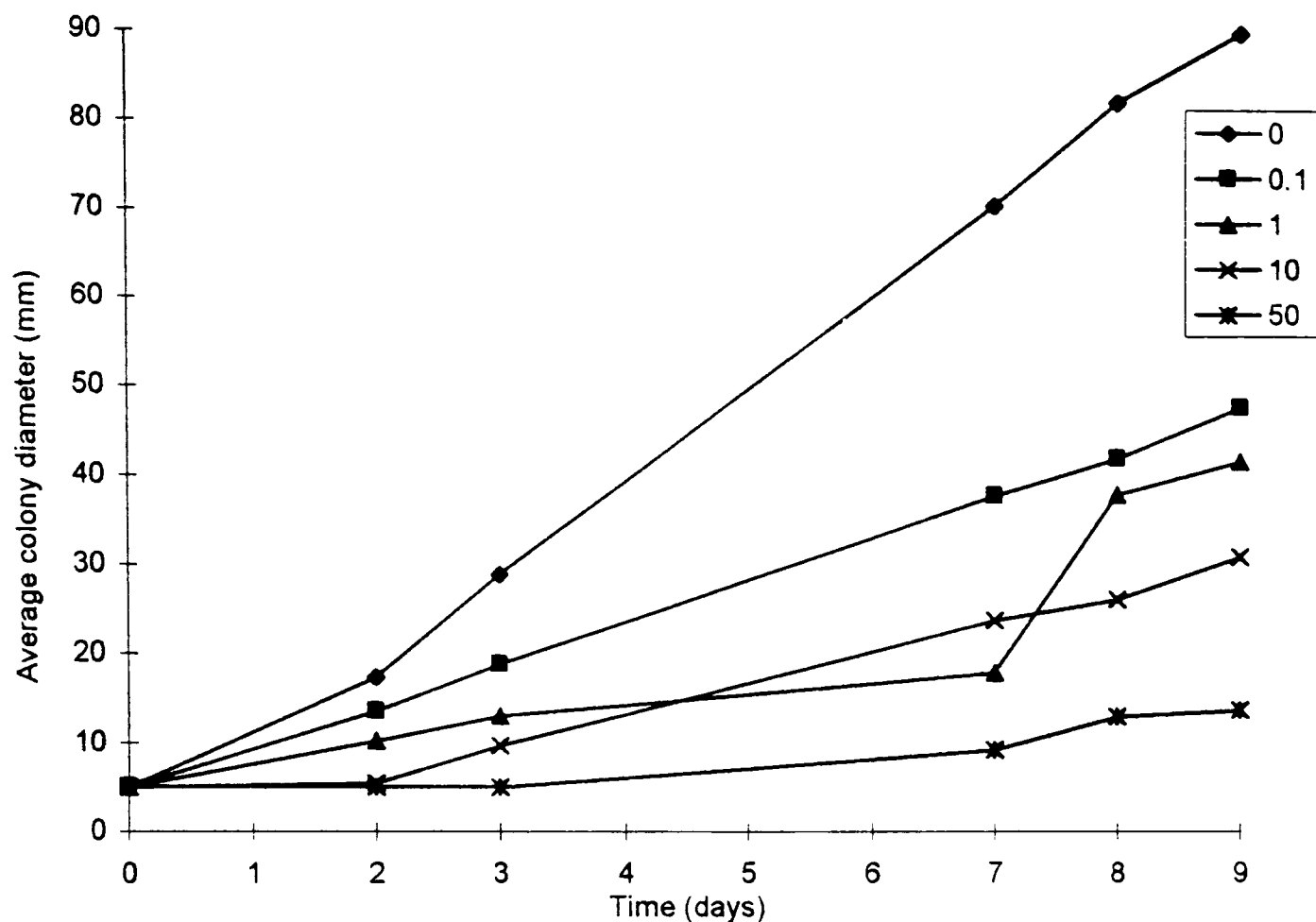


Fig. 1.1.16 Effect of varying concentration (0, 0.1, 1.0, 10.0 and 50.0 $\mu\text{g/ml}$) of flusilazole on the average colony diameter (mm) of five isolates of *F.avenaceum* on six dates. For SEM values see appendix 1.1.16.

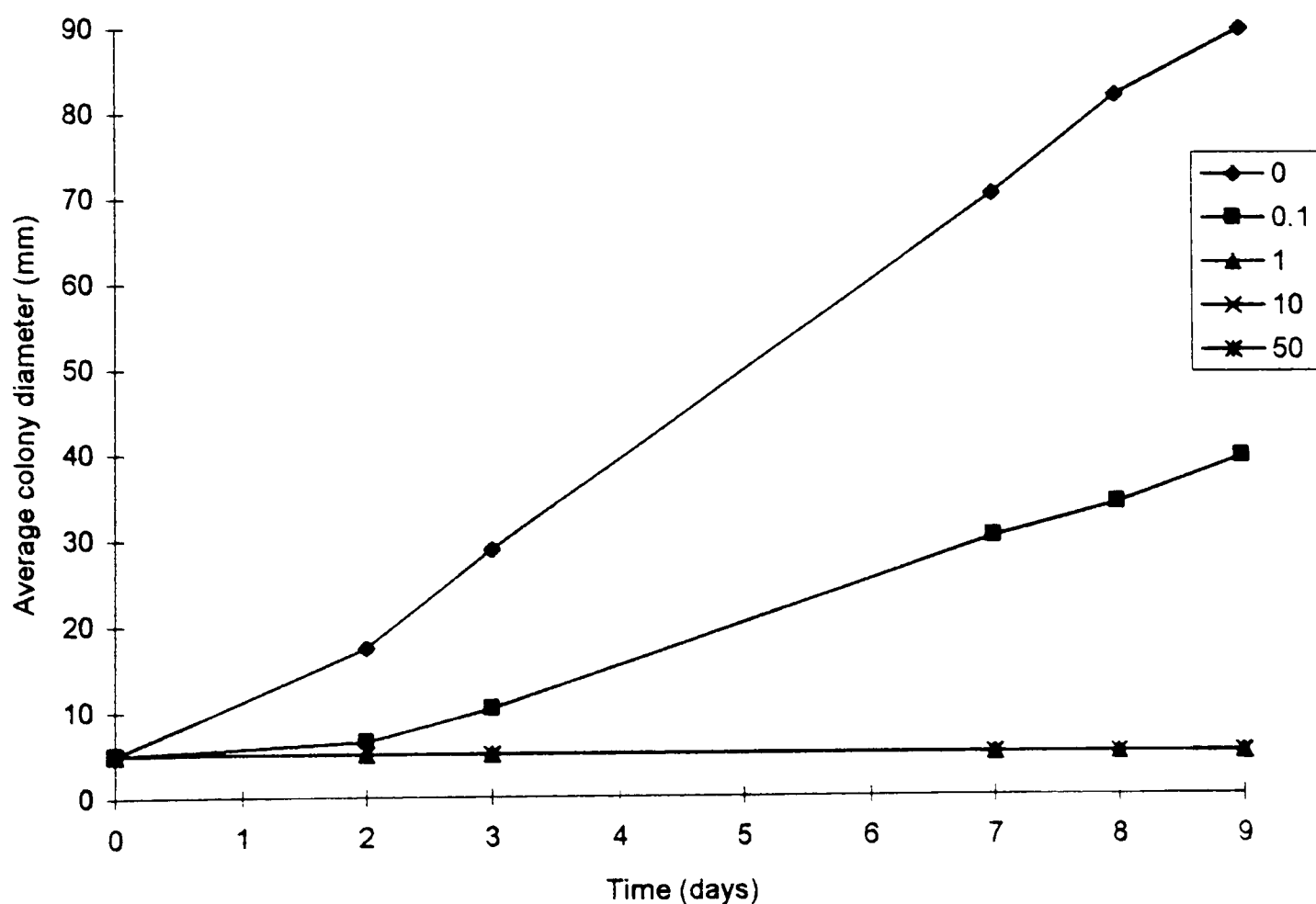


Fig. 1.1.17 Effect of varying concentration (0, 0.1, 1.0, 10.0 and 50.0 $\mu\text{g/ml a.i.}$) of prochloraz on the average colony diameter of five isolates of *F.avenaceum* at six dates. For SEM values see appendix 1.1.17.

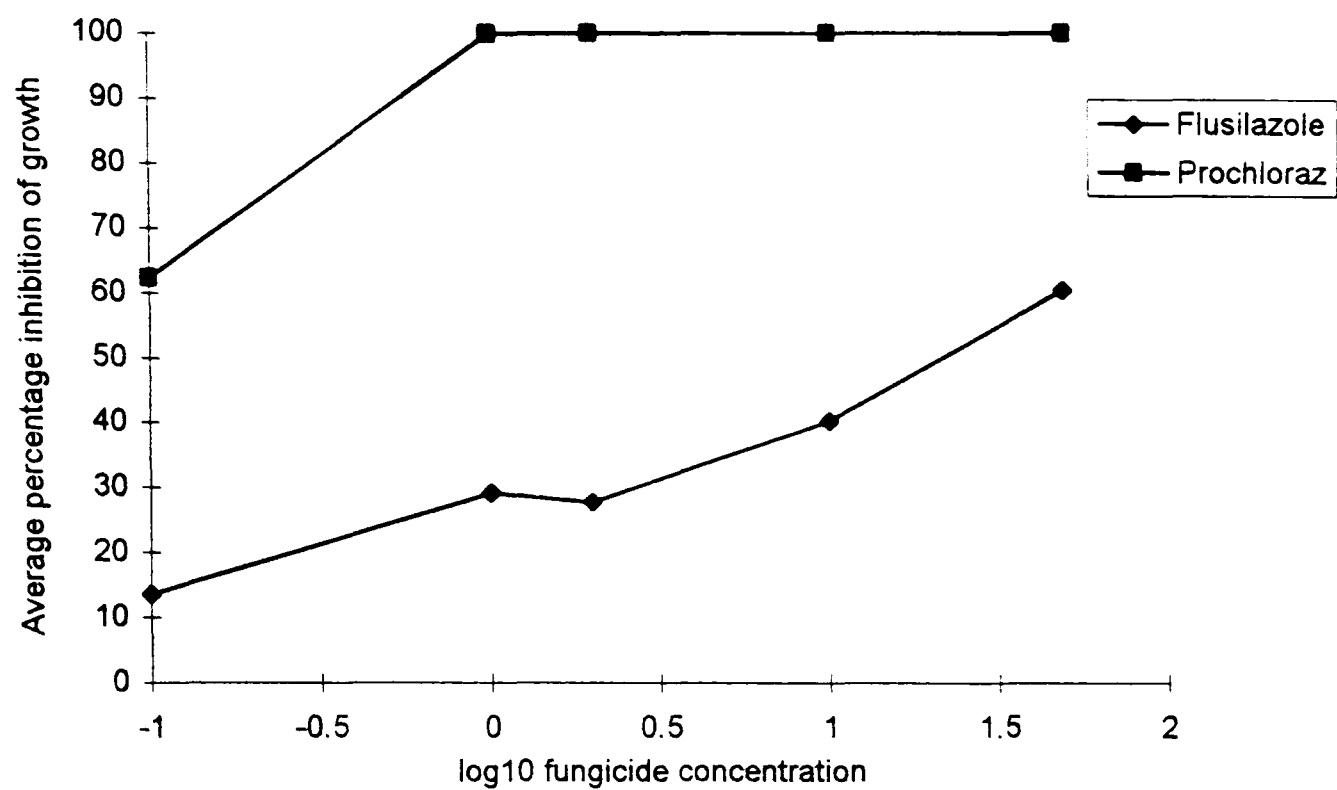


Fig 1.1.18 Effect of varying concentration (0, 0.1, 1.0, 2.0, 10.0 and 50.0 $\mu\text{g/ml}$ a.i.) of flusilazole and prochloraz on the final average percentage inhibition of mycelial growth of five isolates of *F.avenaceum*. For SEM values see appendix 1.1.18.

DISCUSSION

The initial rate of germination of conidia of *M.nivale*, *F.culmorum* and *F.avenaceum* was shown to vary. Whereas conidia of *M.nivale* in the control treatments began to germinate after 3 hours incubation, conidia of *F.culmorum* did not germinate until 9 hours after incubation. *F.avenaceum* was shown to be intermediate between the two types, but tended in general to germinate between 6 and 9 hours incubation.

The initial rate of germination of a conidium may be an important factor affecting the rate of inhibition of fungal germination and mycelial growth caused by fungicides. Pontzen & Scheinpflug (1989) found that the time course of inhibition of sterol biosynthesis by triazole fungicides was different in *Venturia inaequalis*, *Botrytis cinerea* and *Puccinia graminis* f. sp. *tritici* dependent on sterol reserves in the conidium. *P.graminis* did not begin sterol biosynthesis until 6-8 hours after the onset of germination, whereas the other species with no sterol reserve began sterol biosynthesis within 1-2 hours, and were therefore more quickly inhibited by the demethylation inhibiting fungicides. It is possible therefore that the rapid germination of conidia of *M.nivale* compared to *F.culmorum* and *F.avenaceum* resulted in the increased incidence of inhibition of conidial germination by flusilazole and prochloraz in this species. Examination of the sterol biosynthesis of *Fusarium* species and *M.nivale* would be important in examining this hypothesis. The results of the *M.nivale* germination test accord with the findings of Waterfield & Sisler (1989), who showed that germination of sclerotia of *Sclerotium rolfsii* was initially inhibited at a range of concentrations of the triazole fungicide propiconazole, but that only the highest concentration had any effect on germination after 72 hours incubation. These workers suggested that tolerance of sclerotial germination to propiconazole was due to reserves of ergosterol present in the fungus, and that further growth was more sensitive to inhibition due to the high level of accumulation of propiconazole in the hyphae while in the sclerotia.

It was noted, that the conidia of *F.culmorum* when treated with flusilazole, produced more germ tubes which tended to be swollen and stunted. This accords with the findings of Kerkenaar & Barug (1984) who also observed abnormal swelling and branching of germ tube tips in *Ustilago maydis* and *Penicillium italicum* when treated with the DMI fungicides imazalil and fenpropimorph. They attributed these effects to changes in membrane fluidity and activity of membrane-bound enzymes of the fungus due to demethylation inhibition causing abnormal chitin deposition in DMI treated mycelial tips.

The EC50 value of *F.avenaceum* with flusilazole was high in comparison to that of the other species, due to the large amount of variation between isolates of *F.avenaceum* in their response to flusilazole. Variation in the response of the three species tested to the fungicides may be attributable to several factors. Differences in conidial morphology of the three species may play a rôle in this context. The more closely related *F.culmorum* and *F.avenaceum* showed more similar responses to the fungicides than the more distantly related *M.nivale*. The difference in response between *F.culmorum* and *F.avenaceum* to fungicides may also be due to smaller but significant morphological differences between these two species. According to Booth (1971), *F.culmorum* belongs to the Discolor section and *F.avenaceum* to the Arthrosporiella section of the genus *Fusarium*.

Other factors which may be important in influencing the effect of fungicides on pathogens *in vitro* include the growth media, and incubation temperature. For example, Waterfield & Sisler (1989) showed that *Sclerotium rolfsii* was more tolerant to propiconazole when growing on solid medium, than in liquid culture. They also suggested that as colonies get older, the concentration of fungicide at the periphery of the culture may be decreased due to diffusion towards the centre, and accumulation by the older hyphae, or alternatively, that as the fungus grows, the hyphae make minimal

contact with the substrate, and the dose reaching the aerial hyphae is therefore reduced. *F.avenaceum* is in general slow growing, and would therefore have time to accumulate high concentrations of the fungicide in the older hyphae thus increasing the ability of the new hyphae to tolerate lower levels of fungicide. This could explain the increased sensitivity of *F.culmorum* over *F.avenaceum* to flusilazole and prochloraz, and therefore the relatively high EC50 value of *F.avenaceum*. *F.avenaceum* was shown to be in general four times slower growing than *F.culmorum* and much better at tolerating the presence of the MBC fungicide benomyl when grown on amended media (Pettitt pers. comm.).

If growth rates are an important factor affecting the efficacy of fungicides *in vitro*, then the temperature of incubation is probably also an important factor. Species may exhibit different levels of inhibition by fungicides due to the suppression of growth rate by sub-optimal growth conditions. There is a large amount of variation in optimum growth temperatures between isolates of the same species. Optimum *in vitro* growth temperatures were found to be particularly variable in *F.avenaceum*, fluctuating between 15 - 28 °C (Pettitt pers. comm.). There is also a lack of understanding of the interactions between species at different temperatures. For convenience it was therefore necessary to select a temperature suitable for the growth of all three pathogens in this work. From this work it was determined that both flusilazole and prochloraz were able to inhibit the growth and development of *M.nivale*, *F.culmorum* and *F.avenaceum* to varying extents, and that prochloraz was in general more effective than flusilazole in controlling all three species and may therefore be more effective at controlling *Fusarium* foot rot in the field. Once the ability of the fungicides to inhibit the pathogens *in vitro* had been established, it was then possible to examine their efficacy in the field, and to determine whether the discrepancies between *in vitro* and *in vivo* control of fungicides noted by previous workers were applicable in this case.

1.2 The effect of various fungicides, rates of application, spray timings and formulations on the incidence and severity of stem base disease of wheat caused by *F.culmorum*, *F.avenaceum* and *M.nivale*, and the effect on yield.

MATERIALS AND METHODS

Winter wheat (variety Mercia) was drilled at a rate of 375 seeds/m² in 20m² field plots at Harper Adams College in October 1991. The trial was set out according to a randomised block design with four replicates of each treatment. Treatments consisted of a variety of fungicide and wetter formulations and spray timings (Table 1.2.1). The first application of fungicide was carried out on the 3rd December 1991, and the second application on the 9th April 1992. The crop was sampled for *Fusarium* and *Microdochium nivale* symptoms and infections by taking 10 wheat plants at random from each plot. All plants were visually assessed for stem base disease symptoms (see table 1.2.2) at the pre and post growth stage 31 spray date, and at growth stage 75.. The crop was sampled for infection one day before the December and April applications, and 4 weeks after each application. A final sample was also taken at growth stage 75 in mid July.

All stem bases were assessed for infection by *Fusarium* species and *M.nivale*. Stem base sections of length 15-20mm were cut from the main tiller of each plant, placed in sodium hypochlorite solution (5% available chlorine) for 3 minutes and washed in 3 changes of sterile distilled water. After drying on filter paper for 15 minutes, the stem base sections were transferred to potato sucrose agar (PSA) supplemented with 0.1g/l streptomycin sulphate, 0.05g/l chloramphenicol and 0.05g/l neomycin sulphate. Plates were incubated at 20°C in the dark until *Fusarium* colonies emerged. A small plug of mycelium from each colony was transferred to a Petri dish containing sucrose nutrient agar (SNA) (Nirenberg, 1976). A small piece of sterile filter paper was placed on the

surface of the SNA, next to the mycelium in order to enhance sporulation. Plates were incubated under near ultra-violet light (12 light/ 12 hour dark cycle) until sporodochia were produced on the surface of the colony. Conidia were then examined microscopically, and identified using a combination of conidial characteristics and colony morphology according to the method of Brayford (1989). The frequency of *Fusarium* or *M.nivale* species obtained in each sample was calculated as an average from a total of 40 plants sampled per treatment at each date.

All treatments were harvested, and the yield of each treatment calculated in tonnes/hectare as the average of four replicates.

Treatment	Fungicide formulation	Time of application
1	Flusilazole	3/12/91 + 9/4/92
2	Flusilazole (half rate)	3/12/91 + 9/4/92
3	Flusilazole + LI700	3/12/91 (Flus only) + 9/4/92 (Flus+ LI700)
4	Flusilazole + Nuarimol	3/12/91 + 9/4/92
5	Flusilazole + Nuarimol	9/4/92
6	Flusilazole	9/4/92
7	Flusilazole + Carbendazim	9/4/92
8	Prochloraz + Carbendazim	9/4/92
9	Flutriafol	9/4/92
10	Control - no fungicide	-

Table 1.2.1 Fungicide formulations and their times of application to treatments 1-10.

Flusilazole = (400g/l a.i., DuPont (U.K) Ltd, Stevenage, Herts)
 Prochloraz = (400g/l a.i., AgrEvo, Chesterford Park, Essex)
 LI700 (Spraymate) surfactant = (750g/l a.i. Newman Agrochemicals Ltd, Cambridge)
 Nuarimol = (90 g/l a.i., pyrimidine fungicide, Chemtech (Crop Protection) Ltd, Swindon, Wilts)
 Carbendazim = (125g/l a.i. with flusilazole as Punch C, Du Pont (U.K) Ltd)
 = (100 g/l a.i. with prochloraz as Sportak delta, AgrEvo)
 Flutriafol = (125g/l a.i., Zeneca Plant Protection, Haslemere, Surrey)

Area	Score	Description
Nodes	0	No symptoms
	1	Staining on one or more nodes, not covering the whole stem circumference
	2	Staining on one or more nodes, covering the whole stem circumference
	3	One or more nodes rotten, likely to cause lodging
Internodes	0	No symptoms
	1	Slight streaks on stem base
	2	General browning on stem base
	3	Stem base rotted, likely to cause lodging

Table 1.2.2 Foot rot disease scoring system used for nodes and internodes of wheat.

Disease severity index calculated according to Scott & Hollins (1974)

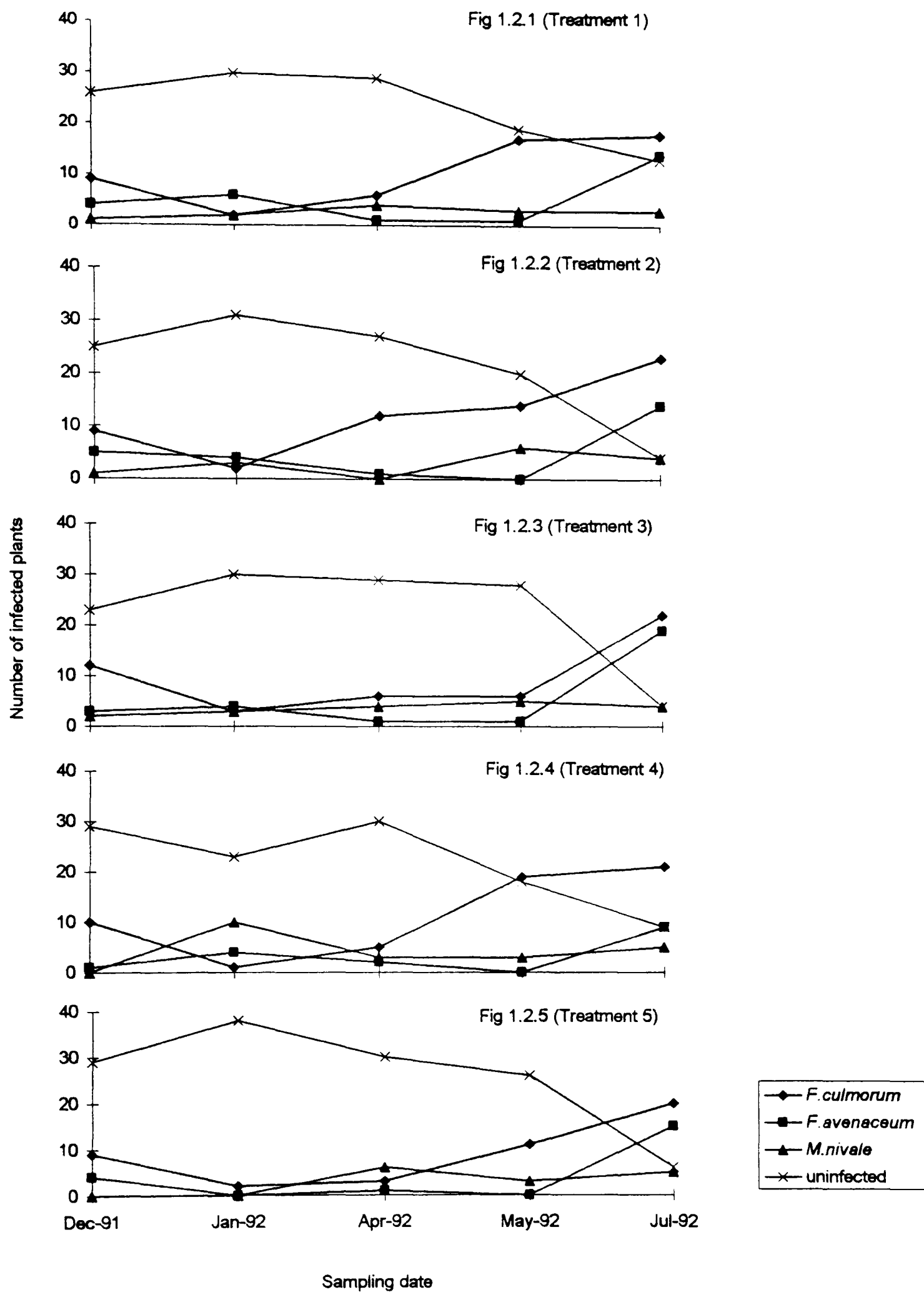
$$\text{Disease index} = \frac{(0 \times a) + (1 \times b) + (2 \times c) + (3 \times d)}{(a + b + c + d)} \times \frac{100}{3}$$

where a = the number of plants with a disease score of 0 etc.

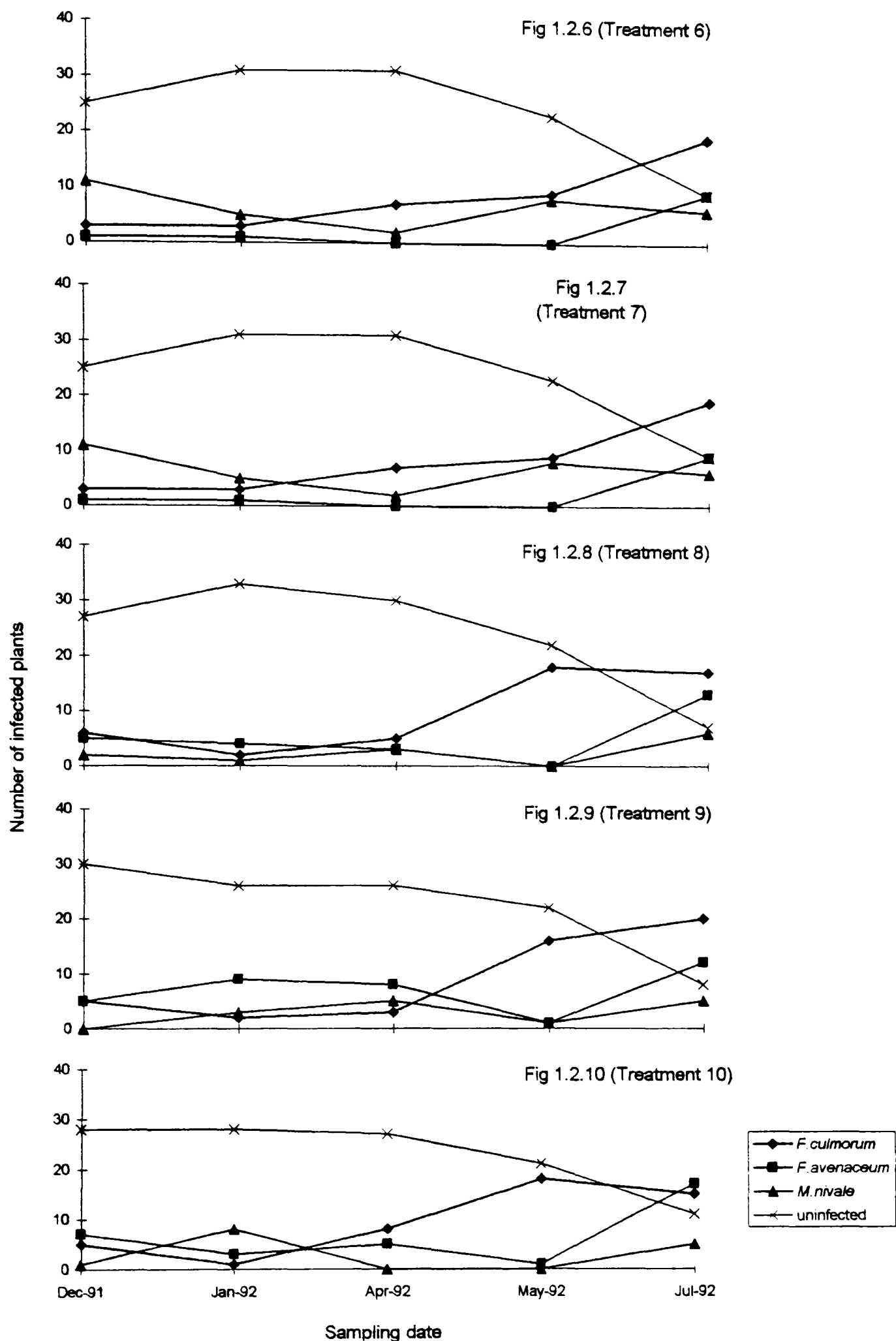
RESULTS

The December and April fungicide applications had no significant effect on the final incidence of *Fusarium* species (Figs 1.2.1-10). The disease scores for pre and post GS31 samples and the GS75 sample showed no significant differences between treatments (Fig 1.2.11), and at GS75 where the variation in disease index between severe disease lesions and uninfected plants was high, the difference between the treatments was minimal. The yields for each of the treatments (mean of four replicates) are illustrated in Fig 1.2.12. There was no significant difference between the yields of any of the treatments.

In the non-fungicide treated control (treatment 10) natural populations of *F.culmorum*, *F.avenaceum* and *M.nivale* were shown to fluctuate over the sampling period (Fig 1.2.10). Even at the early sampling date (December) *F.culmorum*, *F.avenaceum* and *M.nivale* could be isolated from stem base tissue. However, a large proportion of the stem bases examined did not yield *Fusarium* colonies when plated onto agar. At all sample dates the number of *M.nivale* isolations was low compared to *F.culmorum* and *F.avenaceum* (Figs 1.2.1.-10). *F.culmorum* isolations increased continually throughout the season until GS75 when approximately half of the plants sampled were infected with either *F.culmorum* or *F.avenaceum*. None of the fungicide treatments had an effect on the incidence of any of the three species isolated. In all treatments (Fig.1.2.1-1.2.9) the incidence of *F.culmorum*, *F.avenaceum* and *M.nivale* was relatively low until the pre GS31 sample, following which the incidence of all three species increased.



Figs 1.2.1 - 5. Effect of various fungicides, rates of application, spray timings and formulations on the incidence of *F.culmorum*, *F.avenaceum* and *M.nivale* in wheat at five sample dates (40 plants per treatment were sampled, see section 1.2.1). For treatments see table 1.2.1.



Figs 1.2.6 - 10 Effect of various fungicides, rates of application, spray timings and formulations on the incidence of *F. culmorum*, *F. avenaceum* and *M. nivale* in wheat at five sample dates (40 plants per treatment were sampled, see section 1.2.1). For treatments see table 1.2.1.

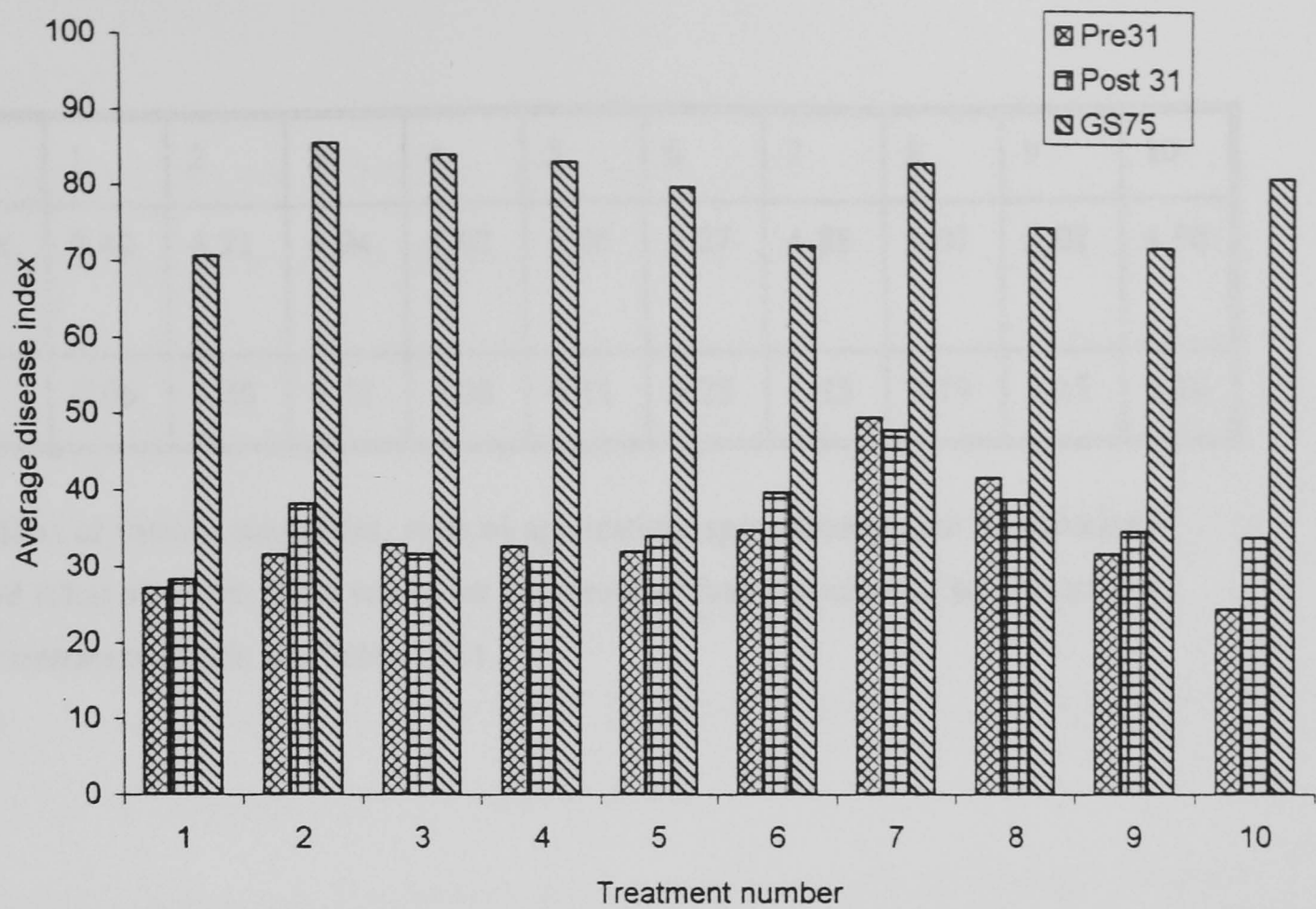


Fig 1.2.11 Effect of various fungicides, rates of application, spray timings and formulations on the *Fusarium* foot rot disease index (average of 40 plants) in wheat at three sample dates (pre growth stage 31, post growth stage 31 and growth stage 75). For SEM values see appendix 1.2.11. For treatment details see Table 1.2.1.

Treatment	1	2	3	4	5	6	7	8	9	10
Average yield (t/ha)	5.42	4.71	4.94	5.02	5.06	5.27	4.88	5.07	5.02	4.90
SEM values	0.06	0.38	0.12	0.38	0.11	0.23	0.15	0.19	0.15	0.16

Table 1.2.3 Effect of various fungicides, rates of application, spray timings and formulations on average yield (t/ha) of wheat. SEM values for the means of four replicates of each treatment are given. For treatment details see Table 1.2.1.

DISCUSSION

In the previous section, differences were noted in the control of *F.culmorum*, *F.avenaceum* and *M.nivale* *in vitro* by the fungicides flusilazole and prochloraz. Prochloraz was more effective than flusilazole in controlling all three species but both fungicides were able to inhibit the growth of all of the species to some extent. However, no evidence of any control of these pathogens was observed in the field using the same fungicides. The timing of fungicide application, the type of fungicide used and the rate of application had no effect on the incidence of infection by any of the three species, the final disease index, or yield of wheat.

The incidence of *M.nivale*, *F.culmorum* and *F.avenaceum* was independent of treatment. The incidence of the three species increased throughout the season from the low level detectable in early December. This early infection of seedlings could result from seed-borne inoculum in the case of *M.nivale*, and soil-borne inoculum in the case of *F.culmorum* and *F.avenaceum*. Later increases in the incidence of each species as recorded on isolation plates may have arisen from secondary spread of conidia from primary infections. The large increase in the incidence of the pathogens (particularly *F.culmorum* and *F.avenaceum*) at the later dates was probably due to the warmer weather favouring growth of these two pathogens. A similar pattern of infection throughout the growing season was noted by Parry (1990).

Throughout the season the incidence of *M.nivale* was relatively low in all treatments. Many factors, including previous cropping, soil type and temperatures as well as inoculum levels can all have an effect on the incidence of any one of the species, and a low incidence of *M.nivale* could be attributed to any one of these factors. However, Pettitt *et al*, (1993) showed that incidence of *M.nivale* infection in wheat stem base tissue can be underestimated using conventional isolation techniques, as faster growing *Fusarium* species out-compete the pathogen. These workers developed a modified

isolation technique which required the use of a benomyl fungicide amended media to select for *M.nivale* which unlike other *Fusarium* species is generally MBC resistant.

The isolation technique used in this experiment may therefore not provide an unbiased estimation of the species present in the stem base tissue and is time consuming. A more reliable technique, and ultimately one incorporating a quantitative element would be useful for the examination of the stem base pathogens and the effect that fungicides have on those pathogens.

The disease severity index increased at each sample date (Fig 1.2.11) with the greatest increase occurring between May and July. This late increase was probably due to environmental conditions at this time being optimal for fungal development. At the GS75 disease assessment, symptom severity was approximately 70 - 80 in the control plots and all treated plots and there was no effect of treatment on the disease index. The fungicides applied had no effect on symptom development, but as the relationship between infection of any one of the species and the resulting symptom severity is not understood, the fungicides may or may not have had differing effects on the various pathogens involved.

As there was no difference in the species incidence data of the treatments, or in the disease indices it was not surprising that there was also no significant difference between the yields of any of the treatments at the 5% level. Generally low yields across the college farm were attributed to very dry weather conditions during Summer. As no differences in disease severity or yield between control and treated plots were noted in this work, the relationship between symptom severity and yield loss could not be determined, and further work was required to investigate this area. Another complicating factor when studying the rôle of *Fusarium* species in symptom severity and yield loss is the presence of the eyespot pathogen *Pseudocercospora herpotrichoides* which can be found in association with *Fusarium* species in stem base disease lesions

(Bateman, 1995). Difficulties in discriminating between eyespot and *Fusarium* symptoms when making disease assessments may confound disease indices results, and the effect of the *Fusarium* species in isolation on the yield cannot be calculated. The effect of eyespot infection on the results of this field trial are therefore not known, although there was not a high incidence of eyespot symptoms. The lack of correlation between *in vitro* tests of prochloraz and flusilazole and field results could be due to several factors. For example, the fungicides applied in the field may not reach the pathogen in the plant, as suggested by Parry (1990). Alternatively, Bateman (1993) suggested that *Fusarium* species escape many of the selective effects of fungicides applied to control eyespot because of their ability to develop late in the season from a soil reservoir which is inaccessible to fungicides applied as sprays to the plant. Bateman (1993) found that W-type eyespot was decreased more by prochloraz than R-type, and suggested that increases in sharp eyespot and brown foot rot where fungicides were applied may have resulted from decreased competition from eyespot. Environmental conditions, and systemicity of fungicides have also been shown to have an effect on their efficacy. Gisi *et al.* (1986) reported that triazoles were less active under cool and humid conditions, and that flusilazole showed a medium level, and prochloraz a low level of systemicity. It is possible that at the concentration of fungicide applied in the field, the effects of the chemical on reaching the fungus are not sufficient to kill it, and that the pathogen can then be successfully isolated from the plant at a later date. The growth of the pathogen may have been suppressed, but this reduction may not be great enough to have an effect on the development and severity of lesions. In addition, suppression of only one of the pathogens may allow the other pathogens present in the disease complex to compete much more strongly, and as it is not known which species cause the most severe disease lesions, it is difficult to monitor the effects of fungicides in this situation.

The trials reported here established that although flusilazole and prochloraz had an

effect on *M.nivale*, *F.culmorum* and *F.avenaceum* *in vitro*, no such effect could be replicated during one years field trial. A large number of factors affecting the efficacy of fungicides operate in the field and before work to examine the impact of these factors can be carried out it was first deemed necessary to address more fundamental questions. Firstly, a reliable system for the identification of different stem base pathogens in the *Fusarium* disease complex was required Quantification of pathogens in plant tissue was also needed, and the effect of *M.nivale* and *F.culmorum*, the major stem base disease components on disease symptom severity and its relation to yield loss in wheat had to be examined further.

2.0 An investigation of the relationship between *Fusarium* and *Microdochium* infection in winter wheat, disease symptom severity and yield loss.

Symptoms characteristic of *Fusarium* foot rot caused by *F.culmorum*, *F.avenaceum* and *Microdochium nivale* include brown lesions on the nodes and internodes of plants. Such symptoms may cause weakening of the stem leading to premature ripening or lodging of the crop, although according to Cook (1980), foot rots are rarely severe enough for this to occur. The relationship between infection of winter wheat by these pathogens and subsequent severity of disease symptoms and yield loss is not well understood. Cook *et al.* (1991) showed that annual disease induced losses in winter wheat in England and Wales (1985-1989) averaged 1.078 million tonnes, but these workers were not able to attribute any of these losses to infection by *Fusarium* pathogens, as no satisfactory yield loss relationships were available. Similarly, Polley *et al.* (1993) were not able to determine losses due to *Fusarium* infection in their survey of cereal diseases in England and Wales during 1981-1991, although work has shown that *F.culmorum* *F.avenaceum* and *M.nivale* can commonly be isolated from wheat stem base disease lesions (Parry, 1990, Polley & Thomas 1991).

The effect of disease severity on yield has however been demonstrated in other cereal pathogens. For example, Slope & Etheridge (1971) found that each 1% of winter wheat shoots with take-all symptoms caused by *Gaeumannomyces graminis* at the beginning of July decreased yield by 0.6% and Polley & Clarkson (1980) showed that severe take-all of winter wheat resulted in a 53-62% reduction in grain dry weight per plant. Similarly Clarkson & Cook (1983) showed that severe infection of winter wheat by *Rhizoctonia cerealis* significantly reduced yield per year, grain number per ear and thousand grain weight by 26, 20 and 11% respectively. Clarkson (1981) examined single plants infected with the eyespot pathogen *P.herpotrichoides* and found that severe

disease reduced yields by 36% and Jones (1994) found a relationship between eyespot disease incidence at growth stage 75 (GS75) in winter wheat grown under field conditions, and yield following the application of the fungicide prochloraz at GS31. He found that each 1% increase in percentage of tillers with severe eyespot caused a corresponding 0.21% decrease in yield. The effect of such fungicide applications on *Fusarium* foot rot has not been widely investigated, but may enable associations between disease severity and yield to be made. According to Thomas (1986) surveys in England and Wales have shown that spray timing for the treatment of diseases in winter wheat is critical, with some crops receiving three badly timed fungicide applications often having as much disease as severely affected untreated crops.

Another potentially important factor affecting *Fusarium* disease symptom severity is the use of fungicide treated seed, which has in some cases proved to be an efficient method of reducing seedling disease caused by infection with *F.culmorum*, *F.avenaceum* and *M.nivale*. The importance of seed treatment has been clearly demonstrated in the field, when poor crop stands and yield losses occurred when untreated seed heavily infected with *M.nivale* was sown (Richardson, 1974). Millar & Colhoun (1969) showed that *M.nivale* caused pre-emergence death when infected wheat seed was sown in the field and mature infected plants showed typical foot rot symptoms including browning of the leaf sheaths. Early work by Simmonds & Scott (1928) reported that organic mercury compounds protected seedlings from attack by *F.culmorum* in greenhouse tests, and Bateman (1977) showed that treatment of wheat seed with phenyl mercuric acetate significantly reduced seedling disease caused by soil borne *F.culmorum*, *M.nivale* and *F.avenaceum* in inoculated soil, and that low germination rates caused by the presence of *F.culmorum* were improved. Jamalainen (1962) reported increased yields of rye, wheat and barley following the use of a variety of seed treatments, but concluded that although seed treatment with organomercury compounds was effective in controlling

Microdochium nivale when the fungus was seed-borne, it did not always protect seedlings from fungal infection by *M.nivale* during the winter. This work was supported by that of Cook (1968) who suggested that the greatest losses due to disease were caused by foot rot and root rot of wheat arising from soil-borne rather than seed-borne *F.culmorum* inoculum, and that organomercurials were unlikely to provide protection from this type of infection. It is possible that hyphae remain in the seed after treatment with organomercury fungicides and that this provides a source of inoculum for the later development of foot rot disease. For example, Bateman (1983) reported an incomplete control of seed-borne *M.nivale* by organomercury fungicides which appeared to result from part of the infection being deeply sited and inaccessible to the fungicide.

The withdrawal of organomercury as a seed treatment in 1992 underlined the need for investigation of other fungicides for control of *Fusarium* species. Work has shown (Bateman, 1979) that triazole/MBC fungicide mixtures give some control of seed borne Fusaria, but evidence for the widespread incidence of MBC resistance in *M.nivale* (Locke *et al.*, 1987, Pettitt *et al.*, 1993) now brings into question the efficacy of MBC components of seed treatments to control this species. Other workers (Sturz & Johnston, 1985, Martin & Johnston, 1982) found that carbathiin and triadimenol used as seed treatments did not improve germination of wheat seed infected with *Fusarium*, or increase the weight of seedlings, and Duthie & Hall (1985) reported that carbathiin did not protect winter wheat stem bases against seed-borne *F.graminearum*.

The individual ability of different stem base pathogens including *Fusarium* species and *M.nivale* to infect wheat seedlings, and the effect of competition between these species may be an important factor influencing foot rot disease severity. Bateman (1993) found that apparent interactions between eyespot disease and *Fusarium* infections were more apparent where fungicides had been used, and that R-type eyespot was often associated with infection by *Fusarium* species. He suggested that wheat stem bases weakened or

altered by a primary coloniser are often a suitable substrate for a secondary coloniser, often a *Fusarium* species, which may begin infection at a distance from the original lesion and often not cause distinct symptoms itself. *M.nivale* was found to be both an effective primary and secondary coloniser. Such relationships between species may also be heavily influenced by environmental factors such as inoculum load and temperature, although Rawlinson & Colhoun (1969) noted that no information existed to elucidate the relationship between the level of inoculum of *M.nivale* and the occurrence of disease symptoms on plants.

Initially, the aims of this section of work were to investigate the effect of seed treatment and foliar fungicide application on *Fusarium* symptom type and severity, incidence of *Fusarium* infection, and yield of winter wheat. In this preliminary study, both an organomercury and a pyrrolecarbonitrile seed treatment were compared for their efficacy in controlling the seed-borne *Fusarium* pathogens. The work was then developed to examine the relationship between *Fusarium* foot rot disease symptom severity and yield of wheat by the differential control of the foot rot pathogens using a fungicide regime under semi-controlled environmental conditions. This investigation aimed to create a foot rot disease gradient by sequential applications of fungicide, and to monitor the effects of disease at GS31 and GS75 on yield. In conducting such an experiment however, it has already been shown that many factors can influence results. For example, difficulties in maintaining differences in disease symptom severity between treatment and control plots in the incidence of diseased plants throughout Spring and Summer were shown by Celetti and Hall (1987), who were not able to demonstrate consistent control of *Fusarium* using fungicides. Finally, the effect of inoculum density and time of inoculation with *F.culmorum* and *M.nivale* on foot rot symptom severity and yield was studied.

2.1 The effect of seed treatment and foliar fungicide application on *Fusarium* symptom type and severity, incidence of *Fusarium* infection, and yield of winter wheat.

MATERIALS AND METHODS

Sowing of crop

Winter wheat (cultivar Mercia), treated with either the the phenyl mercury acetate fungicide Ceresol (10-300 g mercury/litre, Zeneca Crop Protection, Haslemere, Surrey) at a rate of 1ml/Kg seed, or the pyrrolecarbonitrile fungicide fenpiclonil, sold as 'Beret' (50g/l a.i., Ciba Agriculture, Whittlesford, Cambs) at a rate of 4ml/Kg seed was drilled on 10/10/91 at a rate of 375 seeds/m² in field plots of size 40m² at Harper Adams Agricultural College, Newport, Shropshire. Treatments were randomised within blocks, and there were three replicates of each treatment. Treatments consisted of sequential foliar applications of the pyrrolecarbonitrile fungicide fludioxonil (code CGA173506, Ciba Agriculture) at a rate of 1 litre/Ha (50g/l a.i.) throughout the season (Treatments are given in Table 2.1.1).

Crop emergence and establishment

On the 5/11/91, crop emergence in the control treatments (treatments 1 and 8, see Table 2.1.1) was calculated as the mean percentage of plants compared to the initial rate of drilling by taking ten counts of 0.5m lengths of row. Plant establishment in these treatments was measured as percentage plant survival on the 17/2/92 using the same method.

Disease assessment

At Growth Stage 31 (GS31) (11/3/92), 25 plants were sampled at random from treatments 1, 2, 3 and 4, (organomercury seed treatment and 0, 4, 2 and 1 applications of fludioxonil) and from treatments 8, 9, 10 and 11 (fenpiclonil seed treatment and 0, 4, 2 and 1 applications of fludioxonil) and each was assessed for foot rot symptoms using a *Fusarium* disease key (Table 2.1.2). Sampling of the crop was carried out again at growth stage 75 (GS75) (12/7/92), when 25 plants were taken at random from all plots and assessed for slight, moderate and severe *Fusarium* symptoms on the nodes and internodes using a more detailed key (Table 2.1.3). From the assessment data at GS75, a disease severity index was calculated for each treatment using the formula below (according to Scott & Hollins, 1974)

$$\text{Disease index} = \frac{(0 \times a) + (1 \times b) + (2 \times c) + (3 \times d)}{(a + b + c + d)} \times \frac{100}{3}$$

where a = the number of plants in disease category 0, b = the number of plants in disease category 1 and c and d represent the number of plants in disease categories 2 and 3 respectively.

Isolation and identification of *F.culmorum*, *F.avenaceum* and *M.nivale* from wheat stem base tissue.

Sections of stem base of length 15-20mm were taken from all main tillers of plants sampled at GS31 and GS75. These sections were treated for three minutes in a sodium hypochlorite solution (5% available chlorine) in an attempt to surface sterilise and then washed in three changes of sterile distilled water. After drying on filter paper, the stem base sections were plated onto Potato Sucrose Agar (PSA) (Johnston & Booth, 1983), amended with 0.1g/l streptomycin sulphate, 0.05g/l chloramphenicol, and 0.05g/l

neomycin sulphate. Plates were incubated at 20°C in the dark for four to ten days until fungal colonies appeared. Mycelium from each putative *Fusarium* or *M.nivale* colony isolated was transferred to a Petri dish containing Sucrose Nutrient Agar (SNA) (Nirenberg, 1976) with a small piece of sterile filter paper on the surface of the media to enhance sporulation. Plates were incubated under near ultraviolet light (12hr light/12 hour dark cycle) until sporodochia were produced. *Fusarium* species and *M.nivale* were then identified from spore and colony morphology according to the method of Brayford (1989).

Yield and thousand grain weight measurements

On 18/8/92, all plots were harvested and the fresh weight of the harvested grain, and the percentage moisture content of the grain in each plot were recorded. Yields were calculated as the weight of grain per treatment at 85% dry matter. Thousand Grain Weight (TGW) was measured by taking the weight of one thousand grains in each treatment (average of three replicates), and was also corrected to its value at 85% dry matter.

Treatment number	Seed treatment	Dates of application of foliar fungicide
1	Hg	Untreated
2	Hg	20/11/91, 17/12/91, 16/1/92, 20/2/92 16/3/92 9/4/92 18/5/92
3	Hg	16/1/92, 20/2/92 16/3/92 9/4/92 18/5/92
4	Hg	20/2/92 16/3/92 9/4/92 18/5/92
5	Hg	16/3/92 9/4/92 18/5/92
6	Hg	9/4/92 18/5/92
7	Hg	18/5/92
8	Fen	Untreated
9	Fen	20/11/91, 17/12/91, 16/1/92, 20/2/92, 16/3/92, 9/4/92 18/5/92
10	Fen	16/1/92, 20/2/92 16/3/92 9/4/92 18/5/92
11	Fen	20/2/92 16/3/92 9/4/92 18/5/92
12	Fen	16/3/92 9/4/92 18/5/92
13	Fen	9/4/92 18/5/92
14	Fen	18/5/92

Table 2.1.1 Fungicide treatments and dates of application to a crop of winter wheat (cv. Mercia) during the 1991-92 field trial. Hg = Organomercury seed treatment, Fen = fenpiclonil seed treatment and the foliar fungicide applied at the given dates was fludioxonil.

Description	Category
0	Symptomless
1	Eyespot alone - Honey-brown discoloured area which may have developed into a distinct eye-shaped lesion. There may be a pupil of dark grey to black fungal growth which cannot be easily rubbed off
2	Eyespot + <i>Fusarium</i> . Honey-brown lesions as in 1, but with dark grey or black discolouration around the margins.
3	<i>Fusarium</i> . Charcoal grey discolouration of whole or part of the leaf sheath at the lowest internode.
4	<i>Fusarium</i> . Dark brown discolouration at the margins of the leaf sheath.
5	Vascular discolouration. Indistinct brown vascular discolouration on the leaf sheath.
6	Sharp eyespot alone. Lesions with bleached sometimes shredded centres and thin, well defined reddish-brown margins.
7	Sharp eyespot + <i>Fusarium</i> . Distinct sharp eyespot lesions as in 6, but with dark grey or black discolouration around the margins.

Table 2.1.2 Disease assessment categories 0-7 based on symptoms associated with eyespot (*P.herpotrichoides*), sharp eyespot (*R.cerealis*) and *Fusarium* foot rot assessed at growth stage 31. After Polley *et al* (1991).

SCORE	NODES	INTERNODES
00	No infection	No infection
01	"	Slight streaks on stem base
02	"	General browning on stem base
03	"	Stem base rotted - likely to cause lodging
10	Staining on one or more nodes not covering whole stem circumference.	No infection
11	"	Slight streaks on stem base
12	"	General browning on stem base
13	"	Stem base rotted - likely to cause lodging
20	Staining on one or more nodes covering whole stem circumference	No infection
21	"	Slight streaks on stem base
22	"	General browning on stem base
23	"	Stem base rotted - likely to cause lodging
30	One or more nodes rotted likely to cause lodging	No infection
31	"	Slight streaks on stem base
32	"	General browning on stem base
33	"	Stem base rotted - likely to cause lodging

Table 2.1.3 Stem base disease assessment key used to score wheat stem base disease at growth stage 75, (Polley, 1991).

RESULTS

The average percentage emergence and establishment of wheat seedlings in the control treatments (treatments 1 and 8) was not significantly affected ($p = 0.05$) by seed treatment (see Table 2.1.4).

Of the 1800 tillers from 600 individual plants assessed from treatments 1, 2, 3, 4, 8, 9, 10, and 11 at growth stage 31, only 217 showed stem base disease symptoms. These symptoms fell mainly into categories 3, 4 and 5 (see Table 2.1.5), consisting of grey/brown *Fusarium* symptoms and vascular discolouration. Those treatments which had received four applications of fungicide (treatments 2 and 9) had fewer symptoms in categories 4 and 5 than their controls (treatments 1 and 8 respectively). There were very few eyespot symptoms at this growth stage, and symptomless plants were predominant in all treatments. There was no apparent difference in the incidence of disease in any of the treatments examined in this growth stage 31 assessment.

When stem bases from all treatments were assessed for disease at growth stage 75, symptoms characteristic of infection by *Fusarium* species or *M.nivale* were recorded on 1003 main tillers. When an average disease score was calculated for each treatment (see Fig 2.1.1) there appeared to be a relationship between frequency of foliar fungicide applications and disease severity. Treatment 1, which had received an organomercury seed treatment and no foliar fungicide applications had a disease score of 66.2, which was significantly higher ($p = 0.05$) than that of treatments 2, 3 and 5 which had received the same seed treatment and a total of seven, five and three applications of the foliar fungicide fludioxonil respectively. Although disease scores in treatments 4 and 6 were reduced compared to the control (treatment 1), this reduction was not significant, and treatment 7 which had received only one foliar application of fungicide had a very similar disease index to the unsprayed control (treatment 1).

Similarly, Fig 2.1.1 shows that there was a significant reduction in average disease

scores between treatment 8 (fenpiclonil treated seed only) and treatments 9 and 10 (fenpiclonil treated seed with 7 and 5 foliar fungicide applications respectively) with the mean disease index being reduced from 65.3 in the control (treatment 8) to 44.4 and 48.4 in treatments 9 and 10 respectively. Again, average disease index was reduced in other treatments (11 and 12) which had received fewer sprays. There was no significant difference in the disease scores of treatments according to seed treatment (see Appendix 2.1.1 for analysis of variance).

When the 600 stem base sections which had been assessed for disease at GS31 were plated onto isolation media, 40 isolates of *Microdochium nivale*, 5 isolates of *Fusarium culmorum* and 3 isolates of *F.avenaceum* were obtained (Table 2.1.6). When the disease scores of the plants from which the isolates had been obtained were examined, it was found that a high proportion (24 isolates of *M.nivale* and 2 isolates of *F.culmorum* and *F.avenaceum*) had grown from apparently symptomless wheat tissue (see Table 2.1.5). Twenty one isolates of *M.nivale*, *F.culmorum* and *F.avenaceum* were obtained from plants which had been treated with an organomercury seed treatment and had not received a foliar fungicide application (treatment 1) (see Table 2.1.6), compared to a recovery rate of only one isolate from plants which had received the organomercury seed treatment and four foliar fungicide applications (treatment 2). The number of isolates obtained from treatments where fenpiclonil treated seed had been used was low in all cases independent of the foliar fungicide regime.

Of the 1050 plants assessed for disease at growth stage 75, 1003 showed some type of *Fusarium* symptom. However, only 377 produced colonies of *Fusarium* species or *M.nivale* when plated out onto isolation media (Fig 2.1.2). These isolates were obtained both from stem bases with and without *Fusarium* type symptoms. Of these isolates, 235 (62.3%) were identified as *F.culmorum*, 123 (32.6%) as *M.nivale* and 19 (5%) as *F.avenaceum*. *F.culmorum* was therefore the most commonly isolated species at GS75.

compared to GS31 where *M.nivale* was shown to be predominant. A large proportion of those plants showing moderate disease symptoms at GS75, did not produce colonies when plated out (Fig 2.1.2).

There was no significant difference between grain yields (t/ha) for any of the treatments measured (Fig 2.1.3). The unsprayed control treatments (treatments 1 and 8) had average yields of 5.76 and 6.04 tonnes /hectare respectively, calculated to 85% dry weight (see Appendix 2.1.3 for analysis of variance).

Similarly, no significant differences in mean thousand grain weights (g) were noted in any of the treatments (Fig 2.1.4). For analysis of variance see appendix 2.1.4.

Treatment	Mean percentage plants emerged	SEM	Mean percentage plants established	SEM
Treatment 1 (organomercury)	75.7	2.83	72.0	2.38
Treatment 8 (fenpiclonil)	78.3	0.78	71.8	1.96

Table 2.1.4 Percentage plants emerged (mean of three replicates) on 5/11/91, and established (17/2/92) for treatments 1 (organomercury treated seed) and 8 (fenpiclonil treated seed). Standard error (SEM) values for each measurement are also given.

Disease category	Number of tillers (n=225) in each disease category							
	Treatment number							
	1	2	3	4	8	9	10	11
0 Symptomless	190	217	211	191	183	220	208	170
1 Eyespot	2	0	0	0	1	0	0	0
2 Eyespot & Fusarium	0	0	0	0	0	0	0	0
3 Fusarium (grey)	9	1	1	3	3	0	0	3
4 Fusarium (brown)	12	2	6	23	28	0	4	38
5 Vascular disclouration	12	5	7	5	10	5	13	17
6 Sharp eyespot	0	0	0	0	0	0	0	0
7 Sharp eyespot & Fusarium	0	0	0	0	0	0	0	0

Table 2.1.5 Number of tillers with symptoms in disease categories 0-7 (revised scoring from Polley *et al.*, 1990) for treatments 1, 2, 3, 4, 8, 9, 10 and 11 (for treatments see table 2.1.1) at growth stage 31. A total of 225 tillers were assessed per treatment.

Treatment number (see Table 2.1.1)								
	1	2	3	4	8	9	10	11
Seed treatment	Hg	Hg	Hg	Hg	Fen	Fen	Fen	Fen
Number of fungicide applications at GS31	0	4	2	1	0	4	2	1
Number of <i>M.nivale</i> isolates obtained	22	1	5	11	1	0	0	0
Number of <i>Fusarium</i> isolates obtained	0	0	1	1	1	2	1	2
Total number of isolations	22	1	6	12	2	2	1	2

Table 2.1.6 The relationship between the number of isolates of *Fusarium* (*culmorum* or *avenaceum*) or *Microdochium nivale* obtained from main tillers of wheat at growth stage (GS) 31 and the number of applications of the foliar fungicide CGA173506 (for treatments see table 2.1.1). Hg = organomercury seed treatment, Fen = fenpiclonil seed treatment.

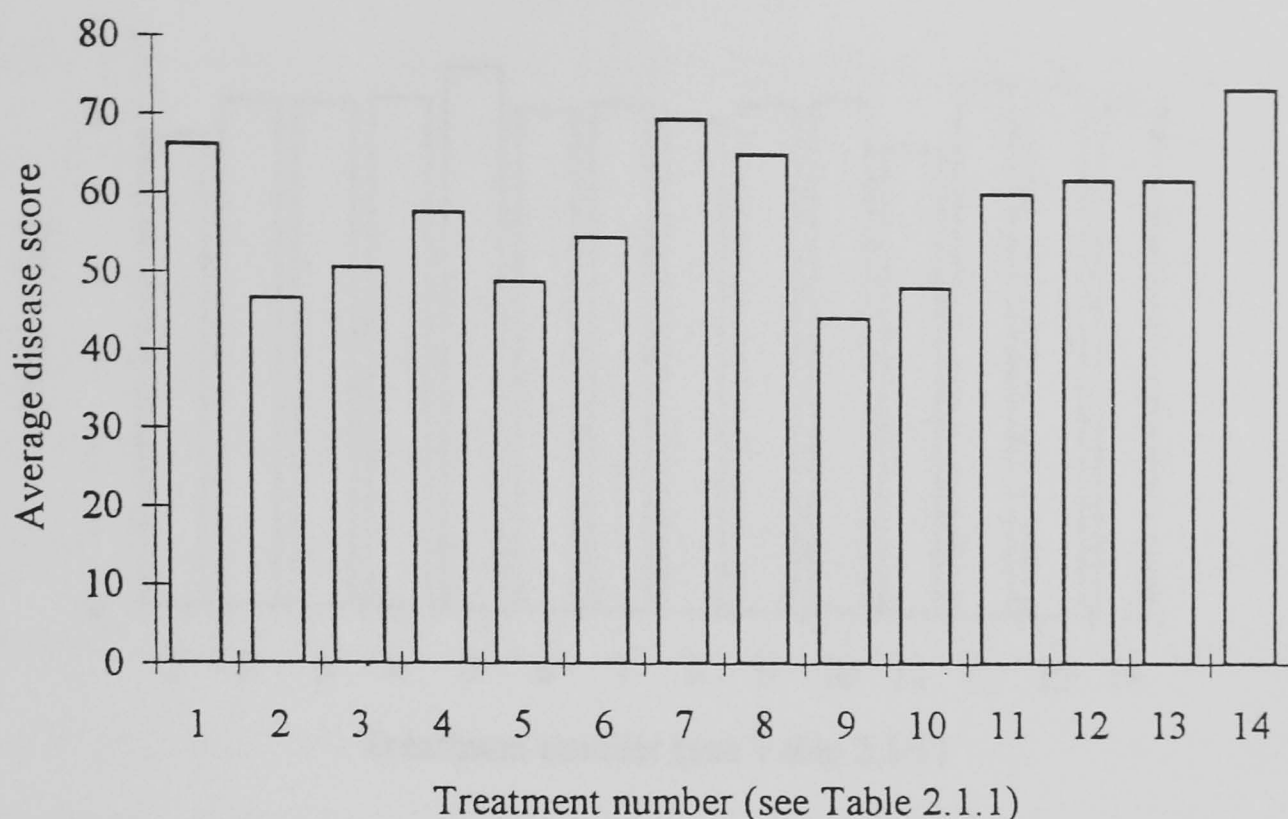


Fig 2.1.1 Effect of two seed treatments (treatment 1-7 = organomercury seed treatment, treatment 8-14 = fenpiclonil seed treatment) and varying applications of the foliar fungicide fludioxonil on the *Fusarium* disease score (average of 75 measurements) on wheat at growth stage 75. (Statistical analysis is given in Appendix 2.1.1).

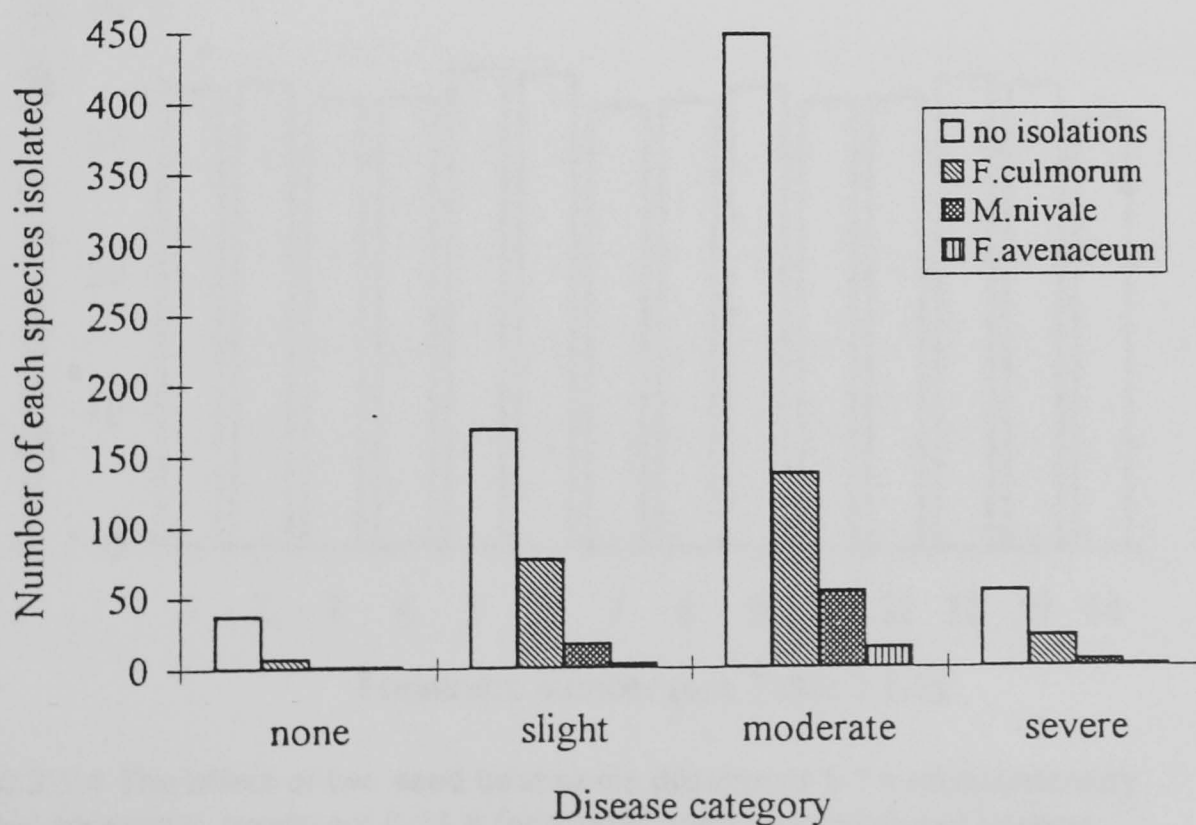


Fig 2.1.2 Relationship between disease symptom category (no disease, slight disease, moderate disease and severe disease) and the isolation of *F.culmorum*, *M.nivale* and *F.avenaceum*. The disease categories correspond to the disease scores given in table 2.1.3 as follows 0 = no disease, 01,10,11 = slight disease, 02,12, 20,21,22 = moderate disease 03,13, 23,30,31,32,33 = severe disease

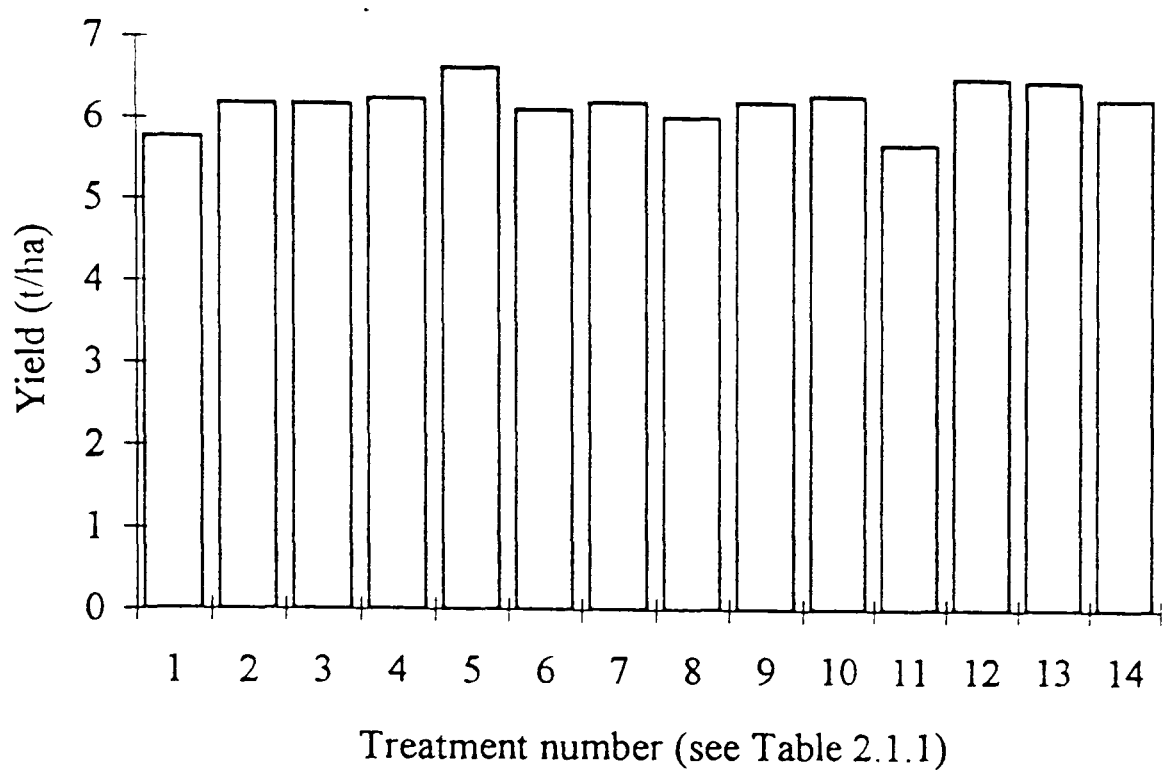


Fig 2.1.3 The effect of two seed treatments (treatment 1-7 = organomercury seed treatment, treatment 8-14 = fenpiclonil seed treatment) and varying applications of the foliar fungicide fludioxonil on the yield (tonnes/hectare, average of three replicates) of winter wheat adjusted to 85% moisture content. (Statistical analysis is given in Appendix 2.1.3).

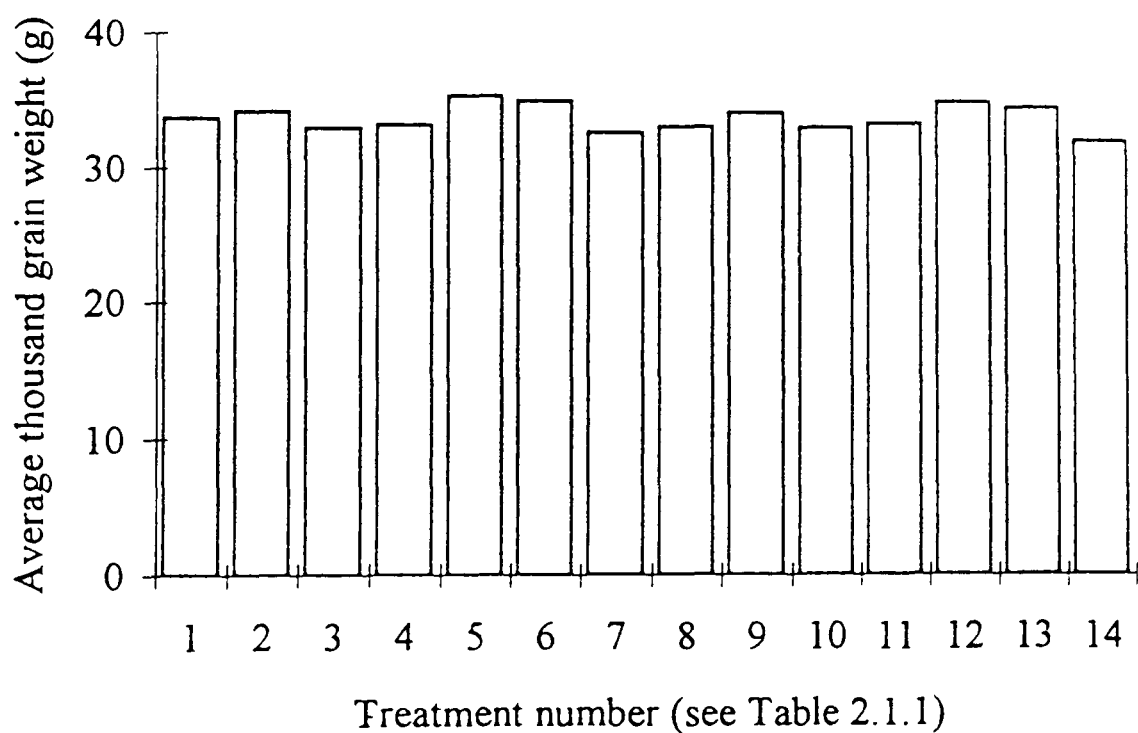


Fig 2.1.4 The effect of two seed treatments (treatment 1-7 = organomercury seed treatment, treatment 8-14 = fenpiclonil seed treatment) and varying applications of the foliar fungicide fludioxonil on the thousand grain weight (grammes, average of three replicates) of winter wheat adjusted to 85% moisture content. (Statistical analysis is given in Appendix 2.1.4).

2.2 An examination of the relationship between *Fusarium* foot rot disease symptom severity and the yield of winter wheat by the differential control of the foot rot pathogens using a fungicide regime under semi- controlled environmental conditions.

MATERIALS AND METHODS

Sowing and maintenance of the crop

Wheat seed (cultivar Mercia), treated with the fungicide Cervax (90g carboxin and 5g thiabendazole/100Kg seed, Zeneca Agrochemicals) was sown in potting compost (John Innes number 3) in large 21 litre plastic containers (600 x 400 x 118mm) at a rate of 84 seeds/container (equivalent to a field rate of 350 seeds/m²) on the 7th November 1992. Containers were arranged in a random block design on a 10 x 10m outdoor gravel surface at Harper Adams Agricultural College. Each block of treatments was surrounded by a row of untreated ('guard') containers and each treatment had 7 replicates, one of which was used only for sampling purposes. Irrigation was provided by a drop irrigation system (Access Irrigation Ltd, Northampton) which supplied equal volumes of water to each container, and was used when required throughout the summer. Nitrogen was applied to each container at a rate of 180kg N/Ha in early April. Treatments consisted of artificial inoculation with *F.culmorum* and/or *M.nivale* with various applications of the fungicides fludioxonil (code:CGA173506, Ciba Agrochemicals, Whittlesford, Cambs) and Sportak delta (AgrEvo UK Ltd, Chesterford Park, Essex). Treatments are shown in Table 2.2.1.

Growth of inoculum and inoculation of seedlings

Four isolates each of *M.nivale* and *F.culmorum* were bulked to provide inoculum. Isolates were cultured on Potato Dextrose Agar (PDA) and incubated at 20°C under a

12 hour light/dark regime for three weeks, or until sporodochia were visible on the surface of the colonies. To produce a spore suspension, the surface of the actively sporulating cultures was washed using sterile distilled water, and the spores were dislodged into this water using a sterile needle. The spore concentration was measured using an haemocytometer, and the volume of this spore suspension required was diluted to provide an inoculum density of 10 000 spores/gram soil in each container. The required volume of spore suspension was diluted in 2 litres of water, and was applied to each container individually using a watering can with a fine rose on 5/2/92.

Fungicide application

The fungicides CGA173506 (C) and Sportak delta (D) were applied at 14 day intervals starting at GS30 at rates equivalent to 1.0 l/ha and 1.25 l/ha respectively. The three application programme of each fungicide (treatments 3, 5, 8, 10, 13, 15, 18 and 20) coincided with the final three applications of the six application programme, and the single spray treatment was co-incident with the final spray of both the three and six spray programmes (see Table 2.2.1).

Growth stage 31 and 75 disease assessment

At GS31, twenty plants were sampled destructively from each of the treatments in replicate block 7 (sampling block) and foot rot symptom severity was assessed using the disease scoring system shown in Table 2.1.3. At GS75, twenty plants were taken from each of the treatments in the remaining six replicates, and foot rot symptom severity assessed using both the nodal and internodal scoring system (Table 2.1.3). A disease severity index was calculated for each treatment at GS31 and GS75.

Isolation of *Fusarium* species and *M.nivale* from stem base tissue.

At GS75, ten plants from those treatments which had received either no fungicide, or the maximum fungicide application (treatments 1, 4, 6, 9, 11, 14, 16, 19 (see table 2.2.1) were assessed for infection by *F.culmorum*, *F.avenaceum* or *M.nivale* using the method of Pettitt *et al* (1993). A 2cm section of stem base tissue was treated in aqueous sodium hypochlorite (5.0% available chlorine) for 5 minutes in an attempt to surface sterilise, and rinsed three times in sterile distilled water. The section was then bisected longitudinally and one half plated onto Potato Dextrose Agar (PDA) containing streptomycin sulphate (100µg/ml), neomycin (50µg/ml) and chloramphenicol (50µg/ml), and the remaining section onto the same medium supplemented with 10µg/ml benomyl (Benlate fungicide, Du Pont U.K. Ltd, 50% w/w a.i.). Plates were incubated at room temperature, and the colonies isolated identified using the method of Brayford (1991).

Yield and thousand grain weight calculations

All ears from each treatment were harvested on 24/8/93. Whole ears were cut off each individual plant using scissors. These ears were then threshed and the grain cleaned using a grain cleaning machine (a/s rationel kornservice, Esbjerg, Denmark). The moisture content (%) of each grain sample was measured using a protimeter. Each sample was then weighed and the yield (g) at 15% moisture content calculated. Thousand grain weight of each sample was also calculated at 85% moisture content by taking the average weight (3 replicates of 250 grains) and multiplying this value by four.

Treatment Number	Species inoculated	Fungicide applications and timings
1	uninoculated	no application
2	"	C (23/5)
3	"	C (25/4, 9/5, 23/5)
4	"	C (14/3, 28/3, 11/4, 25/4, 9/5, 23/5)
5	"	D (25/4, 9/5, 23/5)
6	<i>F.culmorum</i>	no application
7	"	C (23/5)
8	"	C (25/4, 9/5, 23/5)
9	"	C (14/3, 28/3, 11/4, 25/4, 9/5, 23/5)
10	"	D (25/4, 9/5, 23/5)
11	<i>M.nivale</i>	no application
12	"	C (23/5)
13	"	C (25/4, 9/5, 23/5)
14	"	C (14/3, 28/3, 11/4, 25/4, 9/5, 23/5)
15	"	D (25/4, 9/5, 23/5)
16	<i>F.culmorum & M.nivale</i>	no application
17	"	C (23/5)
18	"	C (25/4, 9/5, 23/5)
19	"	C (14/3, 28/3, 11/4, 25/4, 9/5, 23/5)
20	"	D (25/4, 9/5, 23/5)

Table 2.2.1 List of the different combinations of inocula of *F.culmorum* and *M.nivale* and the foliar fungicides fludioxonil (CGA173506) (C) and prochloraz (D) applied to treatments 1 - 20 at given dates (14/3/93, 28/3/93, 11/4/93, 25/4/93, 9/5/93, 23/5/93).

RESULTS

At growth stage 31, disease scores in the uninoculated treatments (treatments 1-5) and in treatments inoculated with *F.culmorum* (treatments 6-10) were low, ranging from 0-15 (Fig 2.2.1). In comparison, the disease scores obtained from those treatments which had received inoculation with *M.nivale* (treatments 11-20) were much higher, ranging from 25-72 (Fig 2.2.1).

At growth stage 75, the average disease scores were high in all treatments (Fig 2.2.2), and there was no significant difference in disease score between the uninoculated treatments, and the *F.culmorum* or *M.nivale* inoculated treatments. Those treatments which had received six applications of the fungicide CGA173506 (treatments 4, 9, 14, 19) did however all show a significantly reduced disease score ($p=0.05$) compared to the other treatments that had received the same inoculum but fewer fungicide applications.

When isolations were carried out in order to ascertain levels of cross contamination between plots at GS75, it was found that control plots were infected with both *F.culmorum* and *M.nivale* (Table 2.2.2). Seven out of ten stem bases assessed from treatment 1 (uninoculated with no fungicide application) and nine out of ten stem bases taken from treatment 4 (uninoculated with 6 fungicide applications) were infected with *F.culmorum*. Each sample also had 2 plants infected with *M.nivale*. The fungicide applications did not therefore appear to have any effect on the incidence of each of these species. In the artificially inoculated treatments, the species isolated predominantly were those that had been applied (Table 2.2.2).

The average yield values ranged from 4.9-7.9 tonnes/hectare (Fig 2.2.3). Treatment four, which received six fungicide applications, and had the lowest average disease score of the uninoculated treatments (treatments 1-5), had a significantly reduced average yield (5.9 t/ha) compared to the other uninoculated treatments. Similarly,

treatment 9 which had been inoculated with *F.culmorum* and had received the maximum fungicide application had a significantly reduced yield compared to the other *F.culmorum* inoculated treatments. No differences in yield were noted in the *M.nivale* inoculated treatments (Fig 2.2.3), and the yields of the mixed inoculum treatments were variable, with treatments 17, 19 and 20 having reduced yields compared to treatment 16, which had received no fungicide. There was no significant difference in thousand grain weight between any of the treatments (Fig 2.2.4)

Treatment number	Species inoculated	Number of fungicide applications	Number of colonies isolated (n = 10)	
			<i>F.culmorum</i>	<i>M.nivale</i>
1	none	0	7	2
4	none	6	9	2
6	<i>F.cul</i>	0	10	2
9	<i>F.cul</i>	6	4	2
11	<i>M.niv</i>	0	1	6
14	<i>M.niv</i>	6	0	5
16	<i>F.cul</i> & <i>M.niv</i>	0	8	6
19	<i>F.cul</i> & <i>M.niv</i>	6	10	9

Table 2.2.2 Number of isolates of *F.culmorum* (*F.cul*) and *M.nivale* (*M.niv*) obtained from wheat stem bases sampled at GS75 from treatments which had received artificial inoculation with *F.culmorum* and *M.nivale*, and either no fungicide, or six applications of the fungicide CGA173506 (Fludioxonil) (treatments 1, 4, 6, 9, 11, 14, 16, 19). Ten plants were sampled per treatment. For a list of treatments see Table 2.2.1.

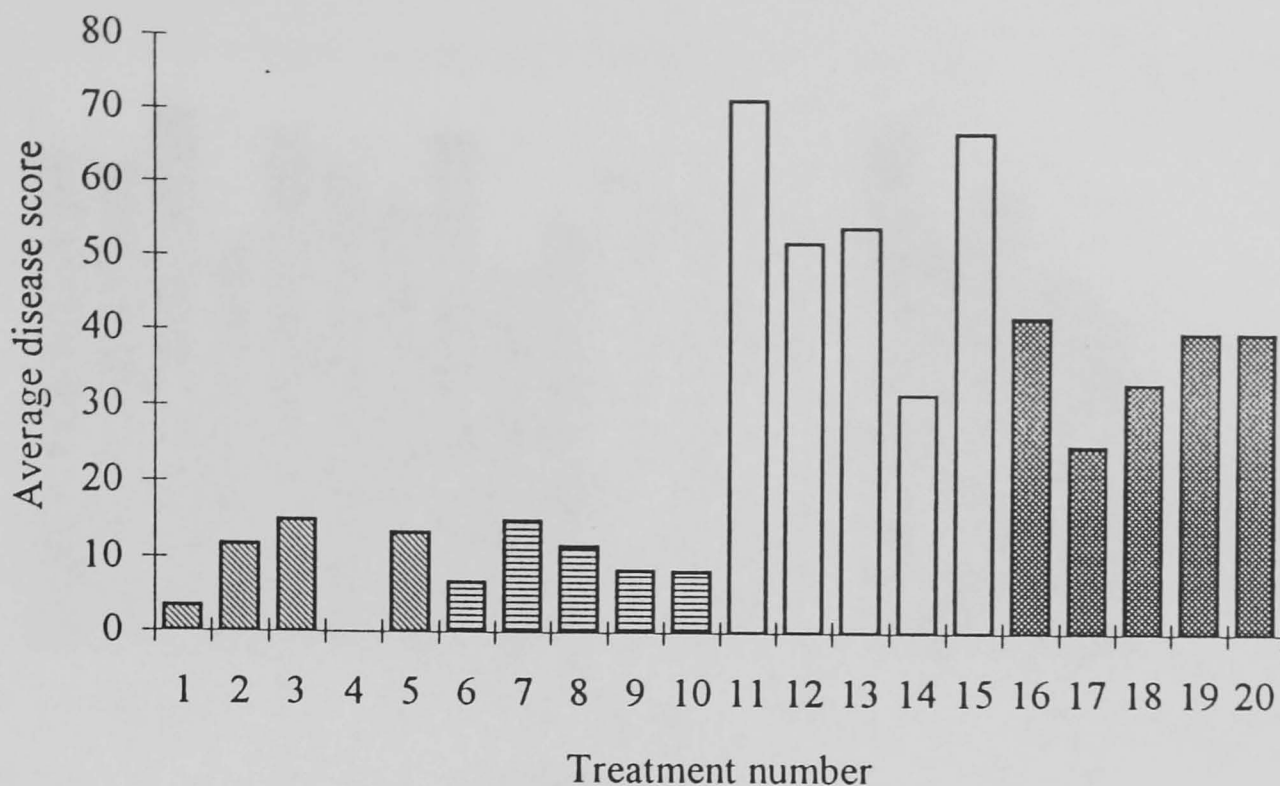


Fig 2.2.1 Disease scores (average of 20 plants) for treatments 1-20 at growth stage 31. ▨ = treatments 1-5 uninoculated, ▤ = treatments 6-10 *F.culmorum* - inoculated □ = treatments 11-15 *M.nivale* - inoculated ▩ = treatments 16-20 *M.nivale* and *F.culmorum* - inoculated. For treatments see table 2.2.1. (Statistical analysis is given in Appendix 2.2.1).

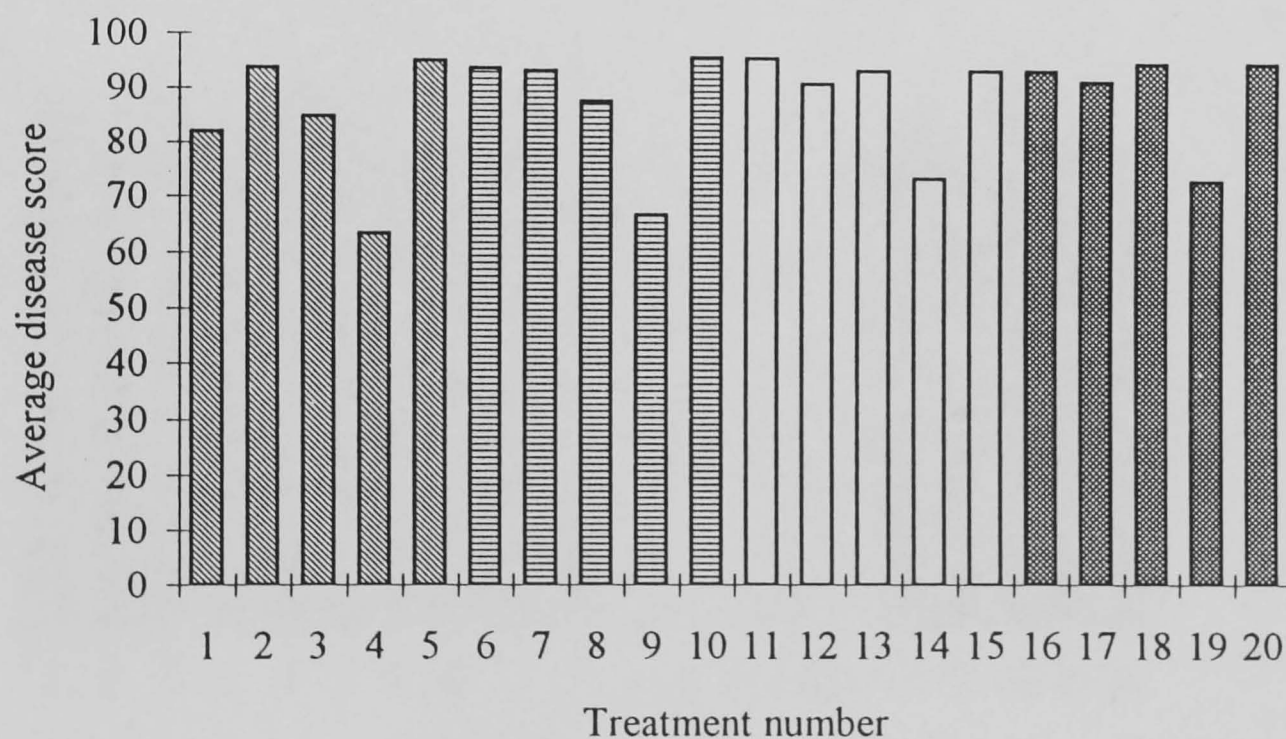


Fig 2.2.2 Disease scores (average of 120 plants) for treatments 1-20 at growth stage 75. ▨ = treatments 1-5 uninoculated, ▤ = treatments 6-10 *F.culmorum* - inoculated □ = treatments 11-15 *M.nivale* - inoculated ▩ = treatments 16-20 *M.nivale* and *F.culmorum* - inoculated. For treatments see table 2.2.1. (Statistical analysis is given in Appendix 2.2.2).

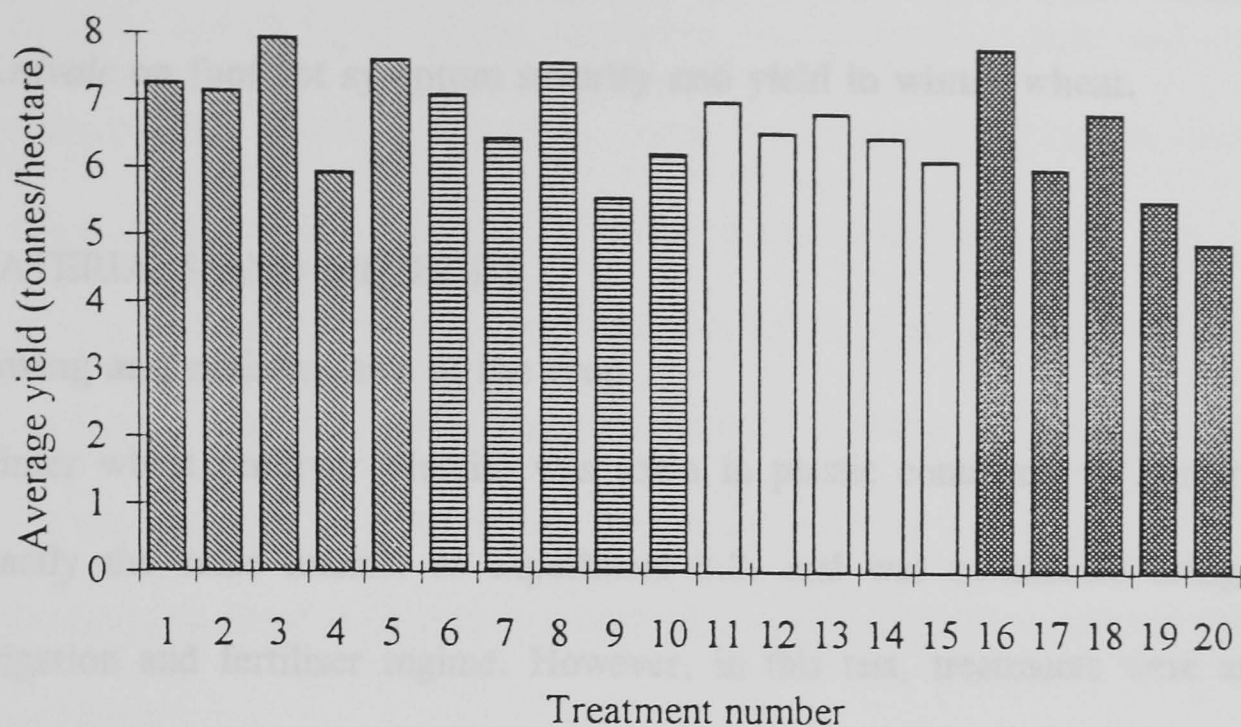


Fig 2.2.3 Yield (tonnes /hectare at 85% moisture content) of wheat cv.Mercia from treatments 1-20 (average of 6 replicates). ▨ = treatments 1-5 uninoculated, ▤ = treatments 6-10 *F.culmorum* inoculated □ = treatments 11-15 *M.nivale* inoculated ▩ = treatments 16-20 *M.nivale* and *F.culmorum* inoculated. For treatments see table 2.2.1. (Statistical analysis is given in Appendix 2.2.3).

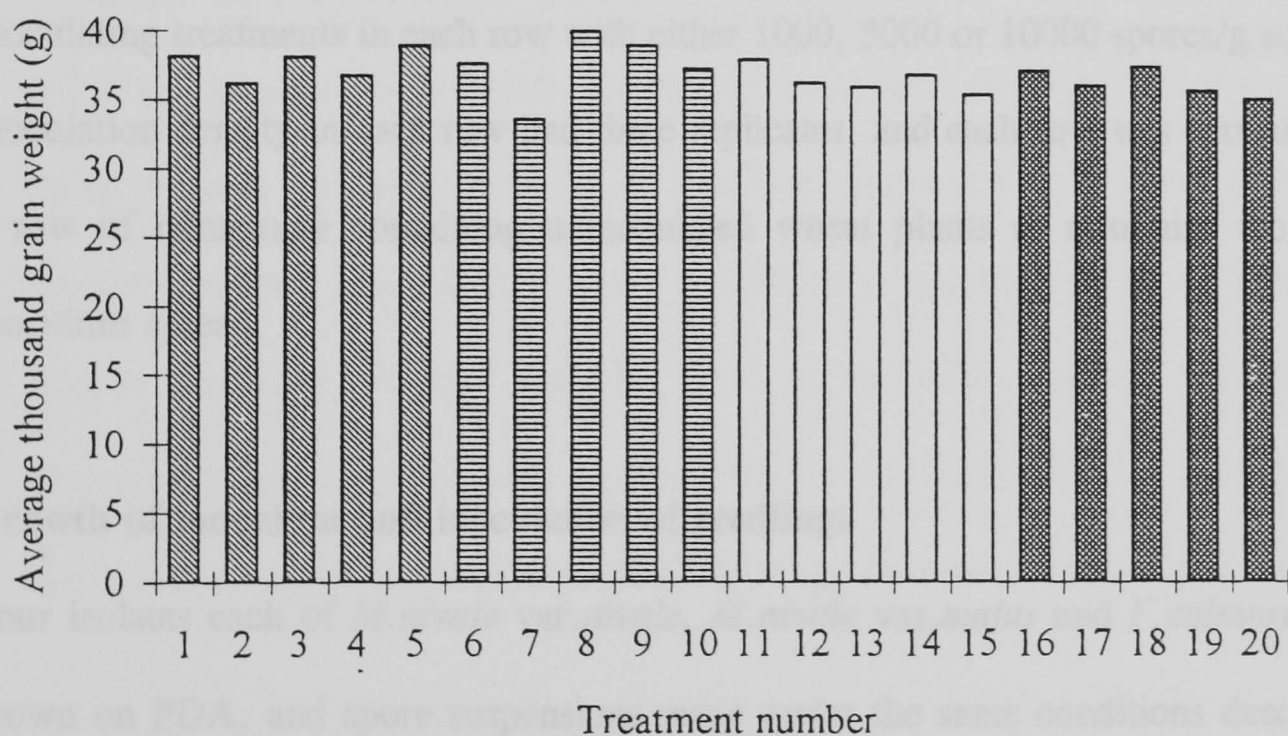


Fig 2.2.4 Thousand grain weight (g at 85% moisture content) of wheat cv. Mercia from treatments 1-20 (average of 6 replicates). ▨ = treatments 1-5 uninoculated, ▤ = treatments 6-10 *F.culmorum* inoculated □ = treatments 11-15 *M.nivale* inoculated ▩ = treatments 16-20 *M.nivale* and *F.culmorum* inoculated. For treatments see Table 2.2.1. (Statistical analysis is given in Appendix 2.2.4).

2.3 The effect of inoculum density and time of inoculation with *F.culmorum* and *M.nivale* on foot rot symptom severity and yield in winter wheat.

MATERIALS AND METHODS

Sowing and maintenance of the crop

Winter wheat (cultivar Mercia) was sown in plastic containers in January 1994 in exactly the same manner as experiment 2.2, and was maintained using the same irrigation and fertiliser regime. However, in this test, treatments were arranged in blocks according to the species with which they were to be inoculated (*F.culmorum*, *M.nivale* or a mixture of the two species). Each block contained three rows, representing three different inoculation timings (March, May and June), and each row consisted of treatments varying in inoculum density. A gradient was created by inoculating treatments in each row with either 1000, 5000 or 10000 spores/g soil. Each inoculation density in each row had three replicates, and each row was surrounded by a row of containers containing uninoculated wheat plants to minimise the risk of inoculum spread.

Growth of inoculum and inoculation of seedlings

Four isolates each of *M.nivale* var.*nivale*, *M.nivale* var.*majus* and *F.culmorum* were grown on PDA, and spore suspensions made under the same conditions described in experiment 2.2. In this case, spore suspensions containing 1000, 5000 and 10000 spores/g of soil of *F.culmorum* or a mixture of *M.nivale* var.*nivale* and var.*majus* were made. Inoculation was carried out as before. Where a mixed inoculum was needed, a full inoculum density of both species was used. For example, if a mixed inoculum density of 5000 spores/g soil was needed, 5000 spores/g of each species was used.

Disease assessment at growth stage 31 and 75

At GS31 and GS75, ten plants were taken from each of the plots, and were assessed for disease using a system of scoring which involved multiplying the lesion length by a value given for each colour of symptom as shown in Table 2.3.1. This adapted method of scoring enabled more information of symptom type to be assessed if necessary. The ten plants from each of the March - inoculated treatments were then taken and analysed for the presence of *F.culmorum* and *M.nivale* using the Polymerase Chain Reaction method described in Chapter 4 at both GS31 and GS75. The results of this test are given in Chapter 4.

Yield and thousand grain weight calculations

All ears from each treatment were harvested on 20/8/94. Whole ears were cut off each individual plant using scissors. These ears were then threshed and the grain cleaned using a grain cleaning machine (a/s rationel kornservice, Esbjerg, Denmark). The moisture content (%) of each grain sample was measured using a protimeter. Each sample was then weighed and the yield (g) at 15% moisture content calculated. Thousand grain weight of each sample was also calculated at 15% moisture content by taking the average weight (3 replicates of 250 grains) and multiplying this value by four.

RESULTS

When *Fusarium* disease symptom severity was assessed in all treatments at Growth Stage 31, those treatments which had previously been inoculated with *F.culmorum* at rates of 1000, 5000 and 10000 spores/g soil had significantly more severe symptoms of *Fusarium*, than uninoculated control treatments (Fig 2.3.1) (see Appendices for statistical analysis). *Fusarium* symptom severity also appeared to be related to inoculum density (Fig 2.3.1), with the disease score in the treatments inoculated with 10000 spores/g *F.culmorum* being higher than that of the treatments receiving less inoculum. Similarly, when the *F.culmorum* - inoculated treatments were assessed for disease at GS75, there was a significant increase in disease symptom severity corresponding to inoculum density (Fig 2.3.2). Those treatments inoculated in March and May had on average higher disease scores than those inoculated in June. The March - inoculated treatments showed a significant increase in disease score when inoculated with *F.culmorum* at 1000, 5000 and 10000 spores/g soil compared to the uninoculated control. There was also a significant increase in disease symptoms between the 1000 and 5000 spores/g inoculum densities, but no such difference between the 5000 and 10000 spores/g disease scores. Likewise, the May inoculated treatments showed significantly more disease at all inoculum densities when compared to the control. The later June inoculation appeared to have less effect on disease severity, however disease was significantly higher in the 1000 and 10000 spores/g treatments than in the control. This increase in disease severity caused by increasing *F.culmorum* inoculum density appeared to be related to a decrease in the average yield of the treatments (Fig 2.2.3). In the March - inoculated treatments, where there was significantly more disease at GS75, the average yield was significantly decreased at a concentration of 1000 spores/g, and further decreased by the higher inoculum concentrations. The May - applied inoculum was less effective in reducing yield despite causing high disease severity at

GS75, and only the highest inoculum density caused a significant reduction in yield. The June applied inoculum had no significant effect on yield at any concentration. Those treatments having received a mixed *F.culmorum* and *M.nivale* inoculum produced similar results to the *F.culmorum* - inoculated plants at GS31, with little disease in the uninoculated treatments, and a significant increase in disease with increasing inoculum density (Fig 2.3.4). At GS75 (Fig 2.3.5) the March and May inoculations produced a significant increase in disease according to inoculum density, whereas the June application only had an effect at the highest inoculum density.

Average yield values for treatments which received the mixed inoculum were generally less affected than those in the treatments which had received *F.culmorum* alone. The March applied inoculum caused a significant reduction ($p=0.05$) in yield at concentrations of 5000 and 10000 spores/g soil, the May application only had an effect on yield ($p=0.05$) at 10000 spores/g of inoculum, and the June application had no effect on yield (Fig 2.3.6).

Similar results were noted in those treatments inoculated with *M.nivale* alone. The GS31 disease assessment showed that the average *Fusarium* disease score was increased significantly by the application of *M.nivale* (Fig 2.3.7) at a rate of 5000 or 10000 spores/g soil. At GS75, disease score was increased by the application of inoculum at 5000 and 10000 spores/g soil in March and May, but no effect on average disease score at GS 75 was noted in the treatments receiving a June application of *M.nivale* (Fig 2.3.8). The application of *M.nivale* inoculum caused a significant reduction in average yield at all concentrations when applied in March. However, there was only a reduction in yield caused by the May - applied inoculum at the highest concentration employed, and the June application had no effect on yield.

There were no differences in yield between the control plots of each of the different treatments.

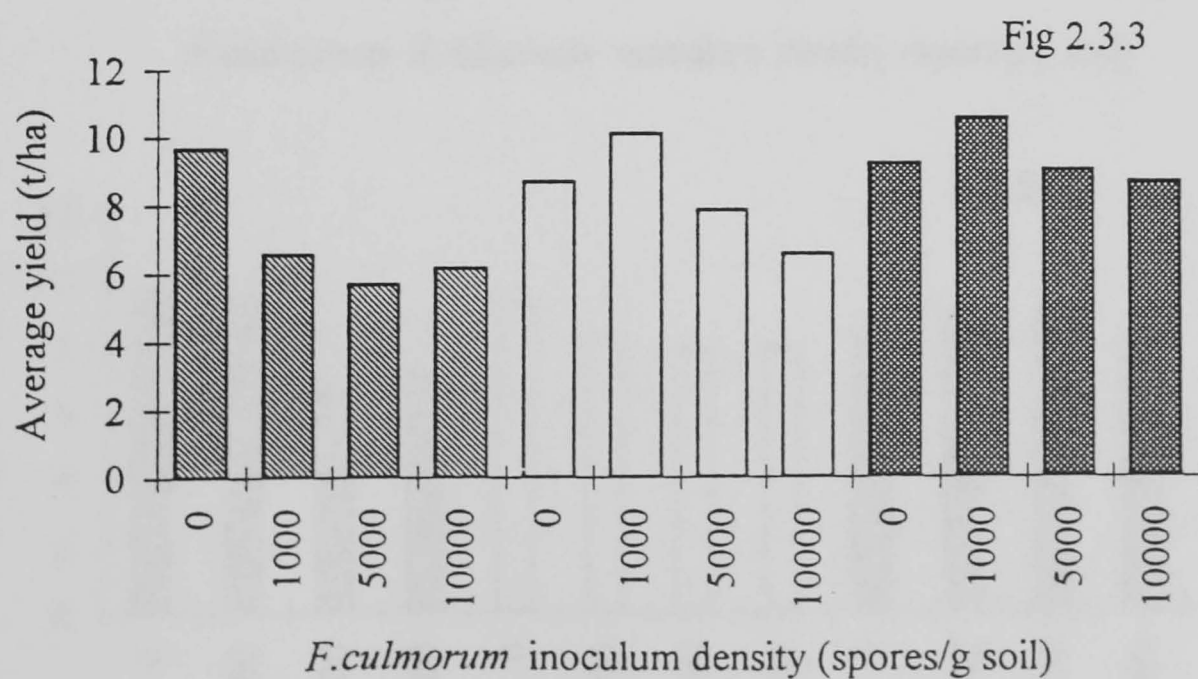
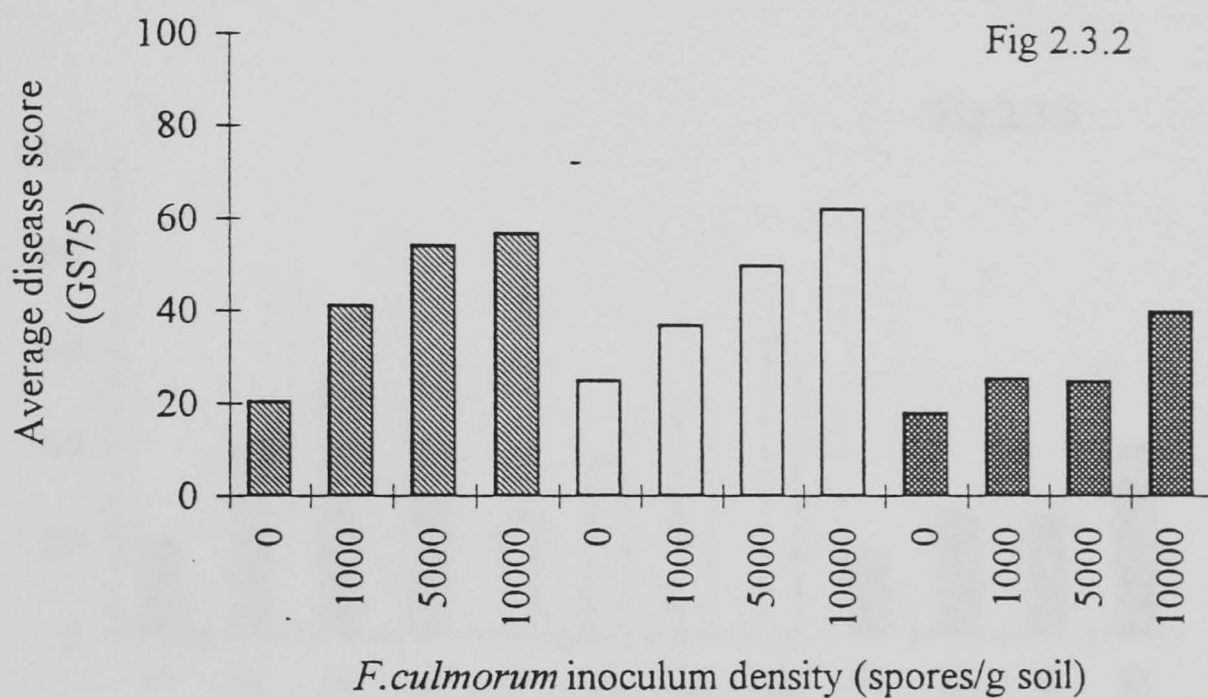
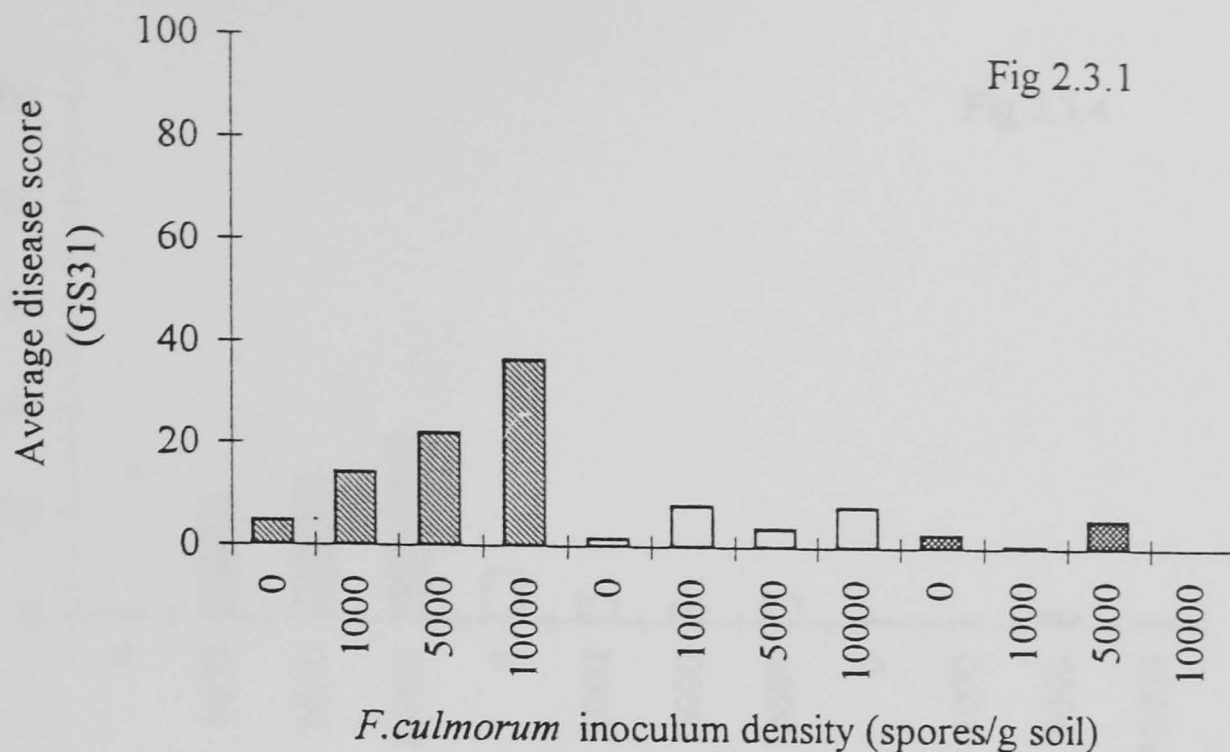


Fig 2.3.1 -3. The effect of inoculation with *F.culmorum* at three concentrations (1000,5000 and 10000 spores/g soil), and at three different times (▨ = March □ = May and ▩ = June inoculation) on the average disease score of winter wheat at growth stage 31 (GS31) (Fig 2.3.1), the average disease score at GS75 (Fig 2.3.2) and the average yield (tonnes/hectare) (Fig 2.3.3). All values are the average of 3 replicates.

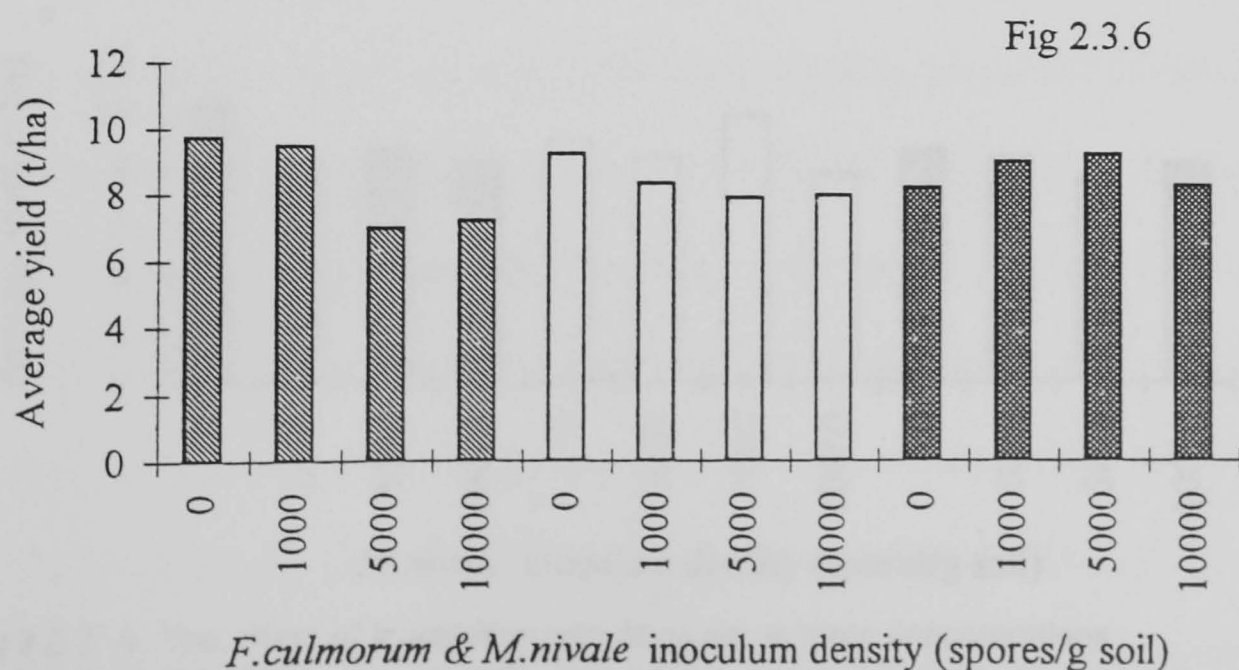
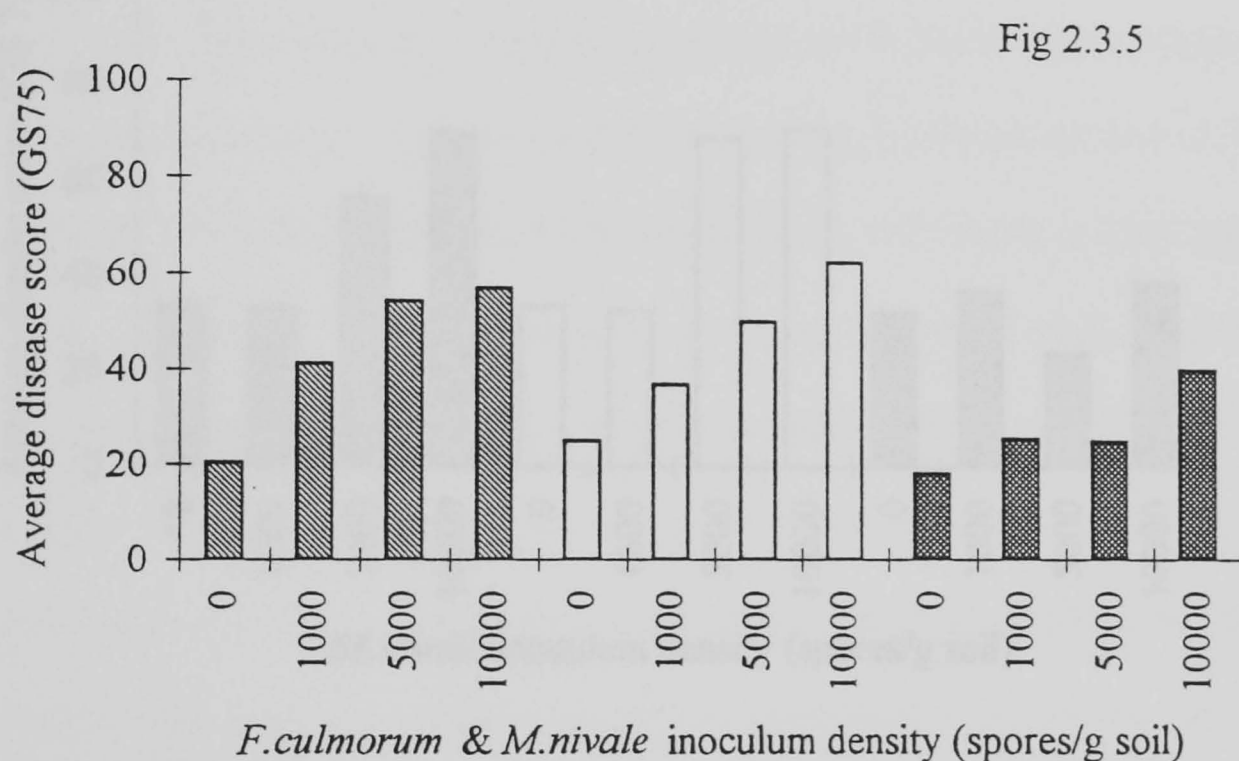
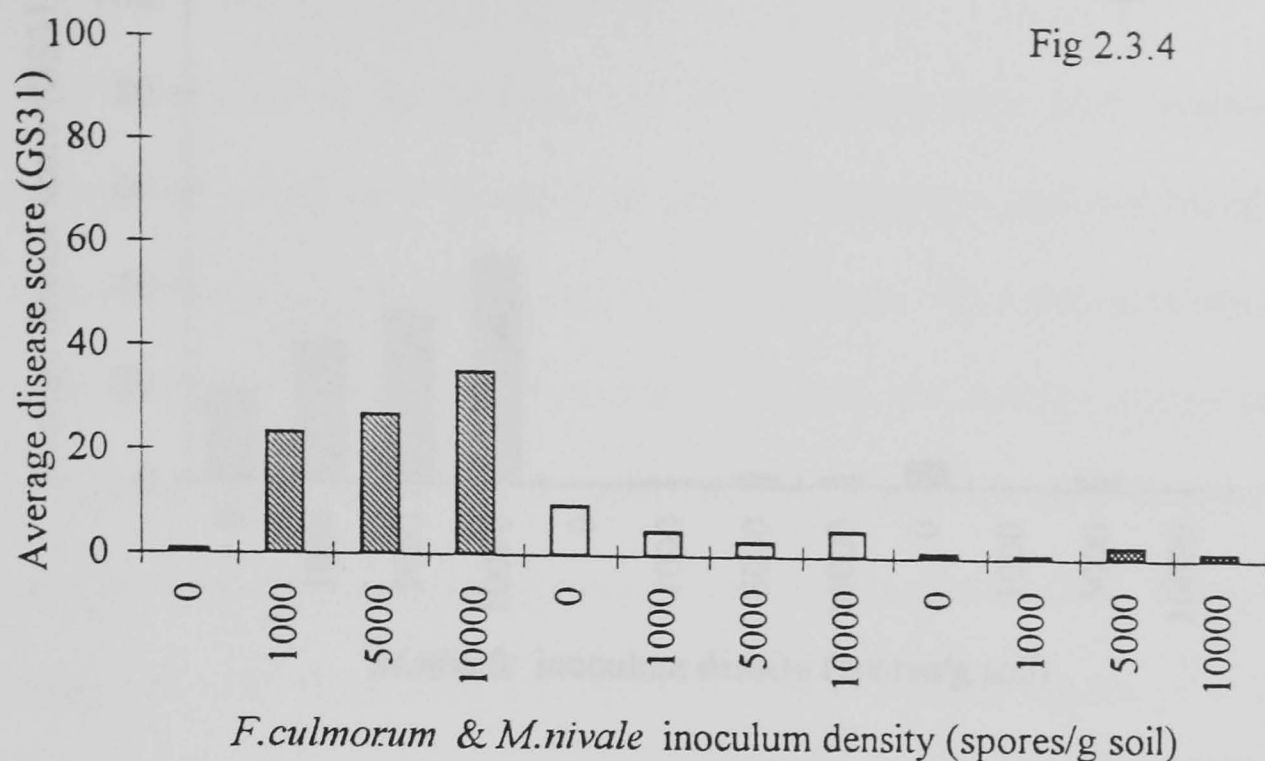


Fig 2.3.4 -6. The effect of inoculation with *F.culmorum* and *M.nivale* at three concentrations (1000,5000 and 10000 spores/g soil), and at three different times (▨= March □= May and ▤ = June inoculation) on the average disease score of winter wheat at growth stage 31 (GS31) (Fig 2.3.3), the average disease score at GS75 (Fig 2.3.4) and the average yield (tonnes/hectare) (Fig 2.3.5). All values are the average of 3 replicates.

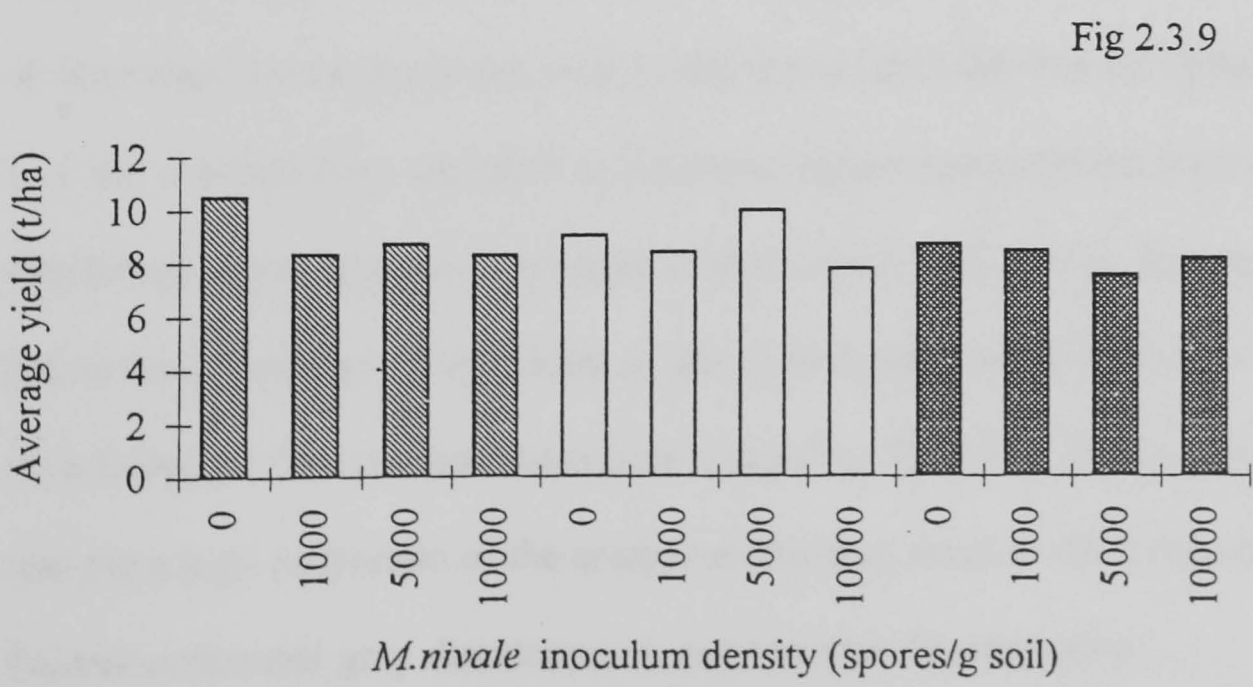
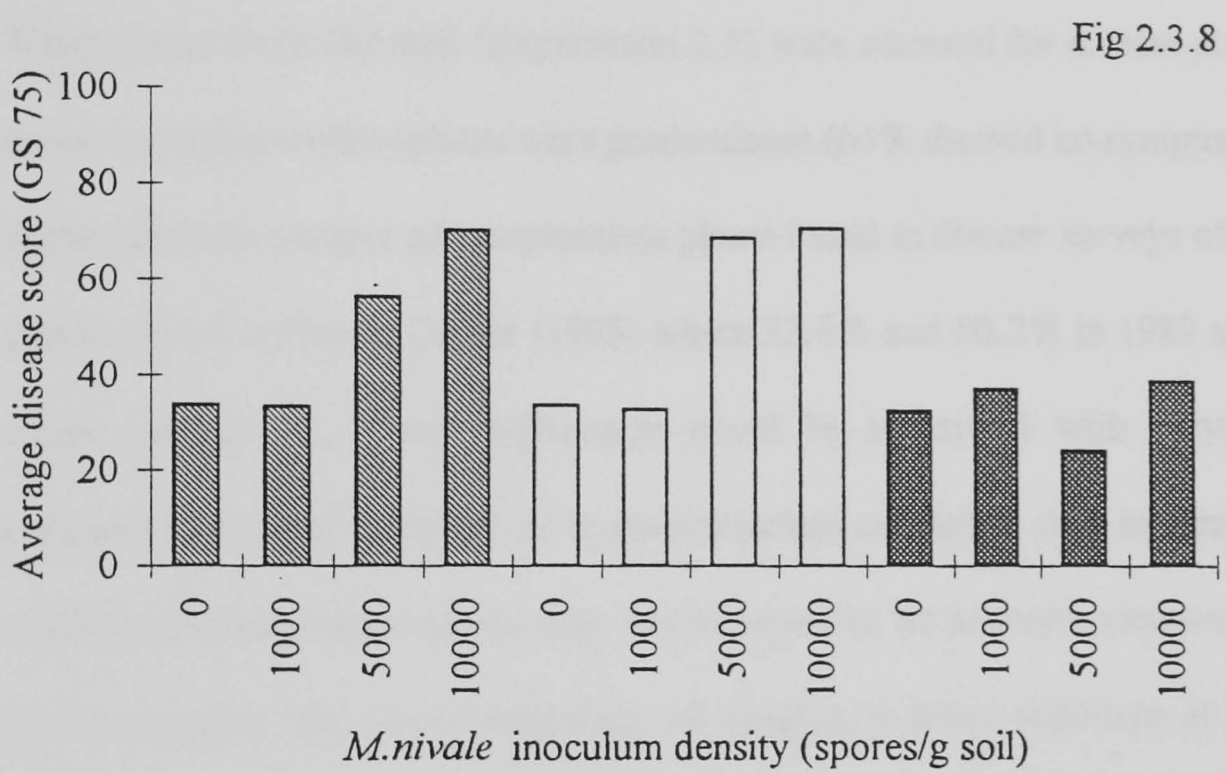
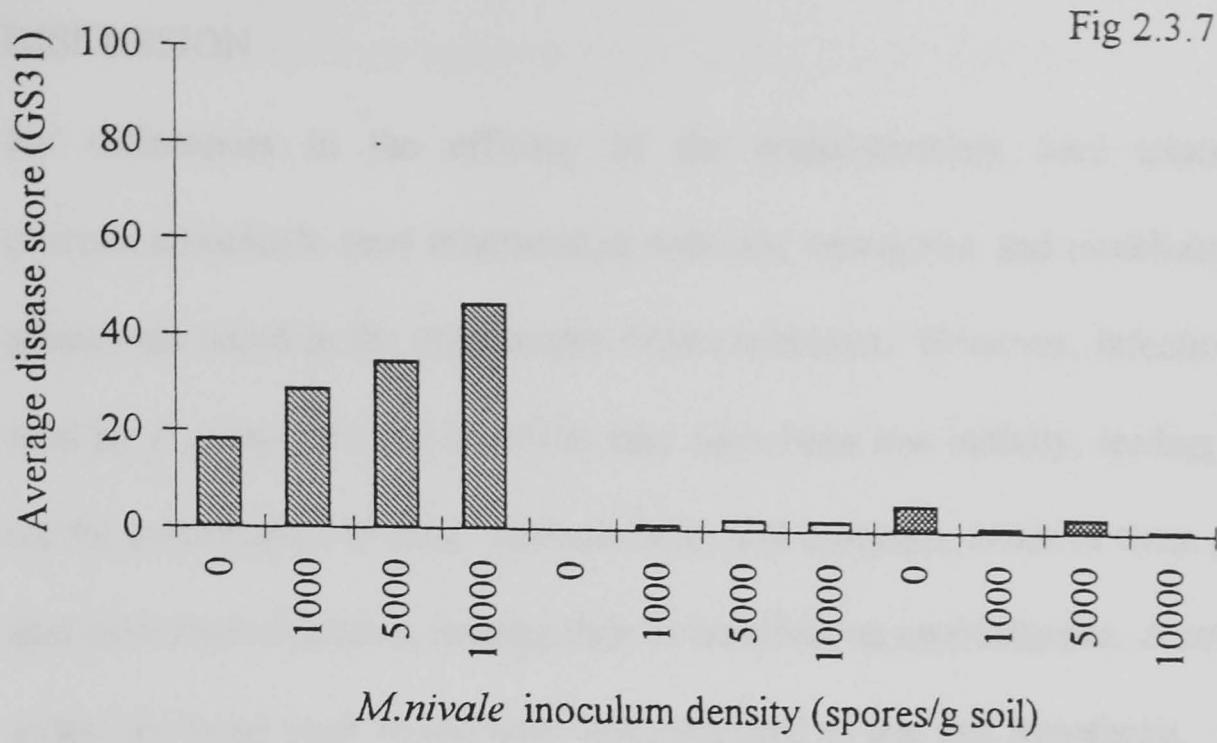


Fig 2.3.7 -9. The effect of inoculation with *M.nivale* at three concentrations (1000,5000 and 10000 spores/g soil), and at three different times (▨ = March □ = May and ▩ = June inoculation) on the average disease score of winter wheat at growth stage 31 (GS31) (Fig 2.3.7), the average disease score at GS75 (Fig 2.3.8) and the average yield (tonnes/hectare) (Fig 2.3.9). All values are the average of 3 replicates.

DISCUSSION

No differences in the efficacy of the organomercury seed treatment and the pyrrolecarbonitrile seed treatment in reducing emergence and establishment of winter wheat was noted in the preliminary field experiment. However, infection of the wheat seed by *F.culmorum* and *M.nivale* may have been low initially, leading to little effect on the germination of seed. Alternatively, soil inoculum levels of these pathogens may also have been minimal, causing little or no effect on establishment. A control treatment using untreated seed would have been required to test this hypothesis.

When plants from this trial (Experiment 2.1) were assessed for disease at GS31, it was found that symptomless plants were predominant (64 % showed no symptoms). This was higher than the number of symptomless plants found in disease surveys of winter wheat conducted by Polley & Turner (1995) where 32.8% and 50.2% in 1989 and 1990 were noted respectively. These differences could be associated with varying inoculum potential in the trials sampled, or to environmental conditions such as temperature. The number of symptomless plants may not however be an accurate measure of infection, as it is possible that minor infections are present in wheat seedlings at this relatively early growth stage and are not expressed as symptoms. It was noted that the majority of stem base disease symptoms seen in this test at GS31 fell into the disease categories 3, 4 and 5 which were described as *Fusarium*-charcoal grey discolouration, *Fusarium*-date brown discolouration and vascular discolouration respectively. This may have been due to the appearance of symptoms at this growth stage being difficult to assess, with most being put into the broadest disease categories. However, Polley & Turner (1995) also put a high proportion of the symptoms that they noted at GS31 into the categories *Fusarium*-charcoal grey discolouration and vascular discolouration.

When the plants assessed for disease at GS31 were plated onto isolation media, only 48 (40 *M.nivale*, 5 *F.culmorum* and 3 *F.avenaceum*) isolates were obtained from a total of

217 stem bases showing apparent *Fusarium* symptoms. This low frequency of isolation could be attributed to inefficiencies in the identification of symptoms, with those symptoms thought to have been characteristic of *Fusarium* actually being caused by the eyespot pathogen *P.herpotrichoides*. Polley & Turner (1995) found that when they made isolations from the lesions which they had assessed visually as joint eyespot and *Fusarium* symptoms, they were able to isolate *P.herpotrichoides* from 48.4% of these lesions, *R.cerealis* from 28.6% and the combined *F.culmorum*, *F.avenaceum*, *F.graminearum* and *M.nivale* isolations constituted only 3.3% of the total. Alternatively, the isolation technique used may have been inefficient. Pettitt *et al.* (1993) showed that the incidence of *M.nivale* in wheat stem base tissue was underestimated using conventional isolation techniques.

A high proportion (24 isolates of *M.nivale*, 2 isolates of *F.culmorum* and *F.avenaceum*) of the isolates obtained from stem base tissue at GS31 were isolated from symptomless plants. This isolation from apparently healthy plant material may have been caused by superficial infection insufficient to cause symptoms, or alternatively the presence of spores in the material which could have produced colonies when plated onto PDA but did not infect the plant. It was noted that *M.nivale* was the most commonly isolated species at GS31. A high incidence of *M.nivale* isolations from wheat stem base lesions at early stages of plant growth was also noted by Parry (1990) and Polley & Turner (1995). In contrast, Bateman (1993) found that early infection of winter wheat by *M.nivale* was rare in assessments carried out in 1989-91. This early infection may be dependent on the amount of *M.nivale* present on the seed or in the soil, the type of fungicide seed treatment, and suitable temperatures for infection and growth of the pathogen, and incidence of *M.nivale* would therefore be expected to be variable between experiments.

When plants in experiment 2.1 were assessed for disease at GS75, 93% were seen to

have *Fusarium*-like lesions at the stem base, with the average severity of these lesions being reduced with increasing application of the fungicide fludioxonil. However, repeated applications of fungicide were required in order to establish this disease control, which brings into question the efficacy of fludioxonil to control the *Fusarium* foot rot in the field. When isolations were attempted from all the stem bases sampled at GS75, only 377 of the 1003 suspected *Fusarium/M.nivale* lesions produced colonies (A recovery rate of 37.5%). However, this was comparable to the recovery rate of isolates from stem base disease lesions shown by Polley & Turner (1995), who were able to isolate these pathogens from 43.5% and 30.7% of disease lesions in 1989 and 1990 respectively. Of the 377 colonies obtained at GS75, 62.3% were identified as *F.culmorum*, 32.6% as *M.nivale*, and 5% as *F.avenaceum*. These results accord with the findings of Bateman (1993), who noted that the development of foot rot symptoms was associated with an increase in the incidence of *F.culmorum* during the summer months of 1989-91. Isolates of *M.nivale*, *F.culmorum* and *F.avenaceum* were again obtained from symptomless plants, which at this later growth stage, where symptoms of infection would perhaps be expected to be more apparent than at GS31 suggests the need for a more reliable method of pathogen isolation.

No differences in yield or thousand grain weight were recorded in this experiment, and therefore it must be assumed that either infection by stem base disease pathogens has no effect on these parameters, or that the fungicide has no ability to reduce disease caused by these pathogens, and therefore no effect on yield can be observed. A major limiting factor in this experiment was an inability to exclude the eyespot pathogen *P.herpotrichoides*, the presence of which could have an effect on the incidence of the other stem base pathogens. Bateman (1993) showed that co-occurrence of different fungi in wheat stem bases was more frequent than would be expected. It is not possible to assess the effects of uneven inoculum potential of each of the pathogens on the

subsequent disease symptom severity in a field situation.

In the subsequent experiment (2.2) where the inoculum potential of the soil was made relatively uniform by the use of compost and artificial inoculation of the required pathogens, the problem of uneven inoculum and infection by *P.herpotrichoides* was largely eliminated.

Booth & Taylor (1976) showed that soil-borne inoculum provided a more regular source of inoculum than the erratic occurrence of seed- borne infection. The incidence of *P.herpotrichoides* was very low due to the absence of infected debris in the soil.

In this case, the GS31 disease assessment scores in the treatments which were uninoculated, or had received *F.culmorum* spores were low compared to those in the *M.nivale* inoculated treatments. This confirms the results of the previous experiment, where *M.nivale* was shown to be prevalent at GS31, and is in agreement with the results of Millar & Colhoun (1969) who showed that *M.nivale* was better suited to cooler conditions than *F.culmorum*.

However, when the treatments were assessed for disease at GS75, all plots showed high disease severities. The control plots were also infected, probably due to contamination by splash dispersal of spores (Jenkinson & Parry, 1994). It is possible that the inoculum load was too high, as the fungicide was only able to reduce disease severity when applied repeatedly. The only effect on yield noted was a reduction in those treatments which had received the highest number of fungicide applications, and which had in fact had a reduced disease score compared to the other treatments. It can therefore be inferred that the fungicide, despite reducing disease caused by *M.nivale* and *F.culmorum* also had a phytotoxic effect on the plant due to its repeated application.

In the concluding experiment (2.3), evidence for a reduction in yield caused by infection of wheat by *M.nivale* and *F.culmorum* was produced. In this case, fungicide was not applied to the experiment, as it was concluded from the previous experiments

that control of the pathogens could not be satisfactorily achieved using any of the fungicides currently available.

When disease was assessed in the artificially inoculated plots at GS31, infection by both *M.nivale* and *F.culmorum* was observed. Disease scores were low in the uninoculated plots at GS31, although some *Fusarium* symptoms were observed. Despite the precautions taken to minimise cross contamination between trays, some disease in the control plots was expected, as seed and soil borne inoculum cannot be completely excluded. However, the aim of the experiment was to create a disease differential between control and inoculated plots, so low disease severities in the control treatments were not considered to be a serious problem. Disease symptom severity at GS31 was also increased by application of increasing inoculum density of *M.nivale* and *F.culmorum* probably due to an increased potential for infection. The effect of increasing inoculum on disease severity at GS31 appeared to become less significant at the highest density, suggesting that a maximum threshold for inoculum density can be reached.

Early inoculation (March) had the greatest effect on disease severity at GS75 independent of the species inoculated, and this was also related to the inoculum density applied. Symptoms were evident on plants assessed at GS75 which had received a late (June) inoculation, although average disease score was generally lower, and was less affected by inoculum density. These results suggest that although late infection by *M.nivale* and *F.culmorum* is possible under these conditions, and can contribute to disease severity, it is the early infections which are responsible for higher yield losses.

Yield was reduced in the treatments inoculated with *M.nivale* and *F.culmorum* according to inoculum density; there was no difference in the reduction in yield caused by the two different pathogens, or by the mixed inoculum. This suggests that despite the predominance of infections by *M.nivale* early in the season, followed by an increase

in *F.culmorum* later in the season, it is possible for both pathogens to infect winter wheat at all stages of the growing season providing that the inoculum density is sufficient, and the environmental conditions favourable for growth. Those treatments which had received the highest inoculum density of *M.nivale* and *F.culmorum*, at the earliest inoculation date, and had the highest disease scores at GS75 also had the lowest yields. These results therefore imply that early detection and eradication of the pathogens could be more effective as a control measure than later fungicide applications administered when disease symptoms were clearly visible.

3.0 Analysis of variation in *Microdochium nivale* from wheat: evidence for a distinct sub-group.

Microdochium nivale (Fries) Samuels and Hallett was originally considered to be a member of the genus *Fusarium* (*F.nivale*), but was later segregated from *Fusarium* (Gams & Muller, 1980) due to its production of annellate conidiogenous cells. It is one of several pathogens including *F.culmorum*, *F.avenaceum* and *F.graminearum* which are able to cause seedling blight and ear blight of cereals, and has been commonly isolated from stem base disease lesions in disease surveys of cereal crops. For example, Rennie *et al.* (1983) isolated *M.nivale* from 80-90% of the wheat stem bases that they sampled, and Parry (1990) found that *M.nivale* was the predominant species isolated in a survey of foot rot on stem bases of winter wheat. Polley *et al.* (1991) also found that *M.nivale* was the most prevalent species isolated from stem bases of wheat at growth stages 31 and 73 (Zadoks, *et al.*, 1974) in 1989 and 1990, independent of symptom severity. Hewett (1983), showed that seed-borne *M.nivale* caused a marked loss in emergence of winter wheat. Symptom type cannot be related accurately to the species causing the disease, although Polley *et al.* (1991) associated charcoal grey lesions with the presence of *M.nivale*.

In the non-taxonomic literature, the occurrence of only one type of *M.nivale* is usually assumed. However, identification of *M.nivale* is based on conidial morphology which has been used to categorise *M.nivale* into 2 varieties. *M.nivale* var. *majus* was named and illustrated by Wollenweber (1931) and reported to differ from *M.nivale* var. *nivale* in having larger conidia. This observation was later confirmed by Gerlach & Nirenberg (1982) who recorded that *Microdochium nivale* var. *nivale* (Fries) Samuels and Hallett was mostly 1-3 septate, whilst *M.nivale* var. *majus* (Wollenw.) Samuels and Hallett was distinguished by wider, predominantly 3 septate conidia. These workers found no

conspicuous differences between the two types in other morphological features.

Noble & Montgomerie (1956) noted that *Monographella nivalis* (Rehm) Muller (the perithecial state of *M.nivale*) was formed freely on infected oat plants in Scotland, and Cook & Bruehl (1966) also found perithecia occurring on wheat plants in the Pacific Northwest of the United States. The rôle of *M.nivale* in causing the snow mould disease of turfgrass was documented by Dahl (1934) and Smith (1983), showed that perithecia of *M.nivale* developed in culture on sterilised cereal straw with isolates obtained from cereals but not with isolates from perennial grasses. He suggested that the *majus* type may produce perithecia more freely, as the turfgrass isolates examined were mainly of the *nivale* type. The only report of the teleomorph of *M.nivale* occurring on grass was made by Dennis (1964). There is no extensive documented evidence concerning the geographical distribution of these two types of *M.nivale*. However, Smith (1981) reported that most of the isolates of *M.nivale* from cereals in Western Canada examined had 0 or 1 septate conidia which indicated that they were mostly the var. *nivale* type, and Harris (1986) found that isolates obtained from a range of gramineous hosts in Australia were also all of the var. *nivale* type.

In a U.K. survey of MBC fungicide resistance in foot rot pathogens, Locke *et al.* (1987) found that 94% of isolates of *M.nivale* tested were resistant to the MBC fungicide benomyl, and Pettitt *et al.* (1993) used MBC amended media to select for isolates of *M.nivale*. However, the response of each variety of *M.nivale* to MBC fungicides has not been examined.

Litschko & Burpee (1987) were unable to differentiate wheat isolates from turfgrass isolates on the basis of conidial morphology, conidiogenesis, response to fungicides and asexual compatibility among thalli, and therefore suggested that distinct biotypes of *M.nivale* did not exist. They also reported sexual crossing between isolates of *nivale* and *majus* classified on the basis of conidial morphology, indicating that the distinction

between the two groups may be of questionable significance. The separation of *M.nivale* into two groups has also been questioned elsewhere (Nelson *et al.*, 1983).

The present work was undertaken to examine variation within *M.nivale* on wheat in order to establish whether *M.nivale* is a single entity, or is composed of sub-groups. In order to examine pathogen populations, and the relationship between the species causing stem base disease of cereals, it is important to clarify the relationship between the two types of *M.nivale*. Additionally, if there is evidence for the existence of these two types, it would be necessary to develop tools for the identification and detection of both, rather than for *M.nivale* as a whole. Evidence for the existence of discrete groups of *M.nivale* was examined in the work described here by comparison of molecular, biological, and physiological markers among a range of isolates from this host.

Molecular markers can be used to examine variation within and between species of fungal plant pathogens (Michelmore & Hulbert, 1987). Examples of the use of restriction fragment-length polymorphisms (RFLPs) include measurement of the relatedness of strains of *Fusarium oxysporum* (Kistler *et al.*, 1987), examination of the relationship between pathogenicity and phylogeny in *Leptosphaeria maculans* (Koch *et al.*, 1991), and assessments of molecular variation within species of *Verticillium* (Carder & Barbara, 1991). In this work, genetic variation among isolates of *M.nivale* from wheat was analysed by Random Amplified Polymorphic DNA (RAPD) assay, and related to the other characters examined. RAPD analysis has been used previously for several purposes, including the differentiation of isolates of *Colletotrichum graminicola* (Guthrie *et al.*, 1992) and *Botrytis cinerea* (Van der Vlugt-Bergmans, 1993), the identification of pathotypes and detection of genetic variation in *Pseudocercospora herpotrichoides* (Nicholson & Rezanoor, 1994), *Leptosphaeria maculans* (Goodwin & Annis, 1991) and *Rhizoctonia cerealis* (Duncan *et al.*, 1993) and the characterisation of races of *Fusarium oxysporum* f. sp. *pisi* (Grajal-Martin *et al.*, 1993). When Correll

et al. (1993) compared methods of examining isolates of *Colletotrichum orbiculare* they found a strict correspondence between mtDNA haplotype, DNA fingerprint group and RAPD group.

This method therefore has the potential to discriminate between isolates of a single species, to highlight the presence of sub-groupings and delineate sub-groupings which exist within the species.

MATERIALS AND METHODS

Origin and maintenance of fungal isolates, and isolation of single spore cultures.

The isolates of *M.nivale* used in this study and their source are given in Table 3.1.1. Isolates were obtained from a range of sites across the U.K., and were isolated from both the stem bases and grains of wheat. Cultures were maintained on Potato Dextrose Agar (PDA) (Difco) plates containing streptomycin sulphate ($100\mu\text{gml}^{-1}$), neomycin ($50\mu\text{gml}^{-1}$) and chloramphenicol ($50\mu\text{gml}^{-1}$) at 15°C .

Sporodochia were removed from cultures of *M.nivale*, and placed in 1.5ml Eppendorf tubes containing $500\mu\text{l}$ of sterile distilled water. Aliquots ($100\mu\text{l}$) of this suspension were removed, and spread on PDA in 9cm Petri dishes. Dishes were left to dry under a laminar flow hood and then returned to a 15°C incubator overnight. Single germinated conidia were located by microscopy under low power (x40), transferred to separate PDA plates, and incubated at 15°C .

DNA extraction, amplification and gel electrophoresis

Single-spore fungal isolates were cultured for 10 days at 15°C on PDA. Mycelium was scraped from the surface of the fungal colonies and added to $500\mu\text{l}$ of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA) in a 1.5ml Eppendorf tube. The tube was placed in

boiling water for 5 minutes, removed and placed on ice. Tubes were then centrifuged for 10 minutes at 13,000 r.p.m. and 2µl of the supernatant used in amplification reactions.

Amplification conditions were identical to those used by Nicholson & Rezanoor (1994). The three oligonucleotide primer sequences (Operon Technologies Inc. Alameda CA94501, US) selected for use with all of the isolates were:

T-14, AATGCCGCAG, V-08, GGACGGCGTT, Y-15, AGTCGCCCTT.

Conidial morphology

All single-spore isolates were plated on PDA and incubated at 15°C illuminated by fluorescent light (Philips TDL 70W/83) under a 16h light/dark regime for 2/3 weeks, or until conidia were produced. Fifty conidia of each isolate were examined and an average spore length, width and number of septa was calculated. On the basis of these characters, all isolates were assigned to one of the two categories as described by Gerlach and Nirenberg (1982).

Production of perithecia in vitro

Straw segments (1cm) were sterilized by autoclaving three times at 121°C for 20 minutes with 24h between each sterilisation. Straws were placed touching along their length in Petri dishes containing PDA and a plug of mycelium from a single spore isolate of *M.nivale* was placed next to the straw. Plates were incubated at 15°C for 4 weeks and the presence of fertile perithecia noted.

Fungicide sensitivity

Petri dishes containing PDA amended with Benlate (Du Pont; 2 mg/l a.i. benomyl) were inoculated with three, 5mm diameter plugs taken from the margin of actively growing

single spore colonies. Following incubation at 15°C for 10 days mycelial growth was assessed by measuring across two diameters, and was compared to growth on unamended PDA.

RESULTS

Primers V08, T14 and Y15 produced RAPD profiles of between 1 and 4 major bands for each isolate; examples of the profiles produced are shown in Fig 3.1.1. Comparison of the fingerprints obtained for isolates of *M.nivale* showed that two distinct groups could be distinguished with primers V08, T14 and Y15. Of the 48 isolates examined, 38 showed very similar RAPD profiles, sharing bands of the same size (Fig 3.1.1). These isolates were categorised as group 1. The remaining 10 isolates (Mn01, Mn30, Mn31, Mn32, Mn33, Mn34, Mn35, Mn37, Mn38, M101) were clearly differentiated from group 1 isolates by all primers. A greater degree of variation was present among these isolates (termed group 2) than among group 1 isolates, as illustrated by primer T14 (Fig 3.1.1).

Conidial morphology was found to differ among isolates in a manner similar to that described by Gerlach & Nirenberg (1982). These workers classified those isolates of *M. nivale* with mostly 1 (0-3) septa, a conidial width of 0-3.8µm and a conidial length in the range 8-27µm as var. *nivale*, and those isolates with predominantly 3 (1-7) septa, a conidial width of 4.2-6.0µm and a conidial length of 15-33µm as var. *majus*.

In this study, average conidial length was distributed continuously in the range 14.3-21.2µm (Fig 3.1.2). Average number of septa was distributed in a similar way to conidial length (Fig 3.1.3), and no distinct groupings could be distinguished using either of these criteria. However, conidial width was distributed broadly into two groups (Fig 3.1.4). Those conidia with a diameter of 4.5µm or less were classified as var.*nivale*, and those with a diameter of 4.9µm or above, as var. *majus*. Of the isolates examined,

9 had on average small ($15.9\mu\text{m}$) narrow ($2.8\mu\text{m}$) conidia, and were assigned to the category *M.nivale* var.*nivale* (Mn01, Mn30, Mn31, Mn32, Mn34, Mn35, Mn37, Mn38, M101). Although isolate M101 was intermediate between the two groups for conidial width ($4.4\mu\text{m}$), it had an average conidial length of $16.4\mu\text{m}$ and on average 1.5 septa, both of which fell into the var.*nivale* grouping. Isolate M101 was therefore classified as var.*nivale*. All 9 isolates classified as var.*nivale* were in the group 2 as defined by RAPD analysis.

Thirty seven isolates had on average longer ($18.4\mu\text{m}$) and wider ($5.0\mu\text{m}$) conidia, and were classified as var.*majus*. Thirty six of those isolates classified as var.*majus* were in the group 1 as defined by RAPD analysis. The other isolate (Mn33), had a conidial width of $4.9\mu\text{m}$ and a length of $18.9\mu\text{m}$, and hence was classified as var.*majus* on the basis of conidial morphology, but had RAPD profiles of group 2 isolates (Fig 3.1.1) which correlated to var.*nivale*. All isolates which originated from seed, and 30 of the isolates (77%) obtained from stem base were classified as *majus* type. Two isolates (Mn17 and Mn48) failed to produce conidia.

Thirty one of the 48 isolates of *M.nivale* examined produced fertile perithecia on wheat straw *in vitro* (see Table 3.1.1). Twenty-four of the 38 group 1 (var.*majus*) isolates and six of the ten isolates within group 2 (var.*nivale*) produced perithecia *in vitro*. There did not appear to be any correlation between group or conidial type and perithecial production *in vitro*.

Only one isolate (Mn30) was sensitive to the MBC fungicide at $2\mu\text{g/ml}$ (Table 3.1.1).

Code	Tissue of origin	Location (county)	Fungicide sensitivity	Perithecial production	Conidial ^b classification	RAPD classification group
Mn01	stem base	Shropshire	-	-	nivale	2
Mn02	stem base	Shropshire	-	-	majus	1
Mn03	stem base	Shropshire	-	-	majus	1
Mn04	stem base	Shropshire	-	+	majus	1
Mn05	stem base	Shropshire	-	+	majus	1
Mn06	stem base	Shropshire	-	+	majus	1
Mn09	stem base	Shropshire	-	-	majus	1
Mn10	stem base	Shropshire	-	+	majus	1
Mn12	stem base	Shropshire	-	+	majus	1
Mn13	stem base	Shropshire	-	-	majus	1
Mn14	stem base	Shropshire	-	-	majus	1
Mn15	stem base	Shropshire	-	+	majus	1
Mn16	stem base	Shropshire	-	+	majus	1
Mn17	stem base	Shropshire	-	+	n.d.	1
Mn18	stem base	Shropshire	-	-	majus	1
Mn19	stem base	Shropshire	-	+	majus	1
Mn20	grain	Borders	-	-	majus	1
Mn21	grain	Borders	-	+	majus	1
Mn22	grain	Borders	-	+	majus	1
Mn24	grain	Shropshire	-	-	majus	1
Mn25	grain	Borders	-	-	majus	1
Mn26	grain	Tayside	-	+	majus	1
Mn27	grain	Tayside	-	-	majus	1
Mn28	grain	Borders	-	+	majus	1
Mn29	stem base	Berkshire	-	+	majus	1
Mn30	stem base	Berkshire	+	-	nivale	2
Mn31	stem base	Berkshire	-	+	nivale	2
Mn32	stem base	Berkshire	-	+	nivale	2
Mn33	stem base	Berkshire	-	+	majus	2
Mn34	stem base	Berkshire	-	+	nivale	2
Mn35	stem base	Berkshire	-	+	nivale	2
Mn36	stem base	Berkshire	-	-	majus	1
Mn37	stem base	Berkshire	-	+	nivale	2
Mn38	stem base	Berkshire	-	-	nivale	2
Mn39	stem base	Berkshire	-	+	majus	1
Mn40	stem base	Berkshire	-	+	majus	1
Mn41	stem base	Berkshire	-	+	majus	1
Mn42	stem base	Berkshire	-	+	majus	1
Mn43	stem base	Berkshire	-	-	majus	1
Mn44	stem base	Berkshire	-	+	majus	1
Mn45	stem base	Berkshire	-	+	majus	1
Mn47	stem base	Berkshire	-	+	majus	1
Mn48	stem base	Berkshire	-	-	n.d.	1
M101	stem base	Norfolk	-	+	nivale	2
M091	stem base	Norfolk	-	+	majus	1
M092	stem base	Norfolk	-	+	majus	1
M112	stem base	Norfolk	-	-	majus	1
M121	stem base	Norfolk	-	-	majus	1

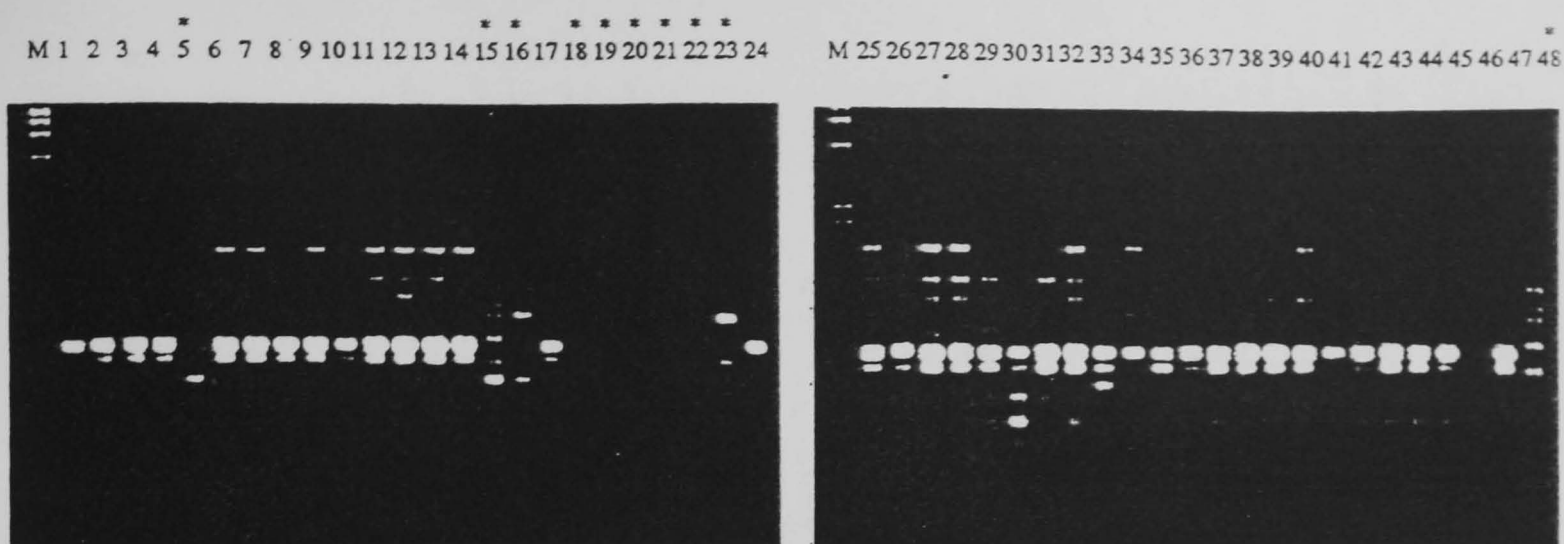
a) Fungicide = 4mg/l Benlate (Du Pont;50% benomyl), + = sensitive - = insensitive

b) Conidia were classified into two groups according to Gerlach & Nirenberg (1982).

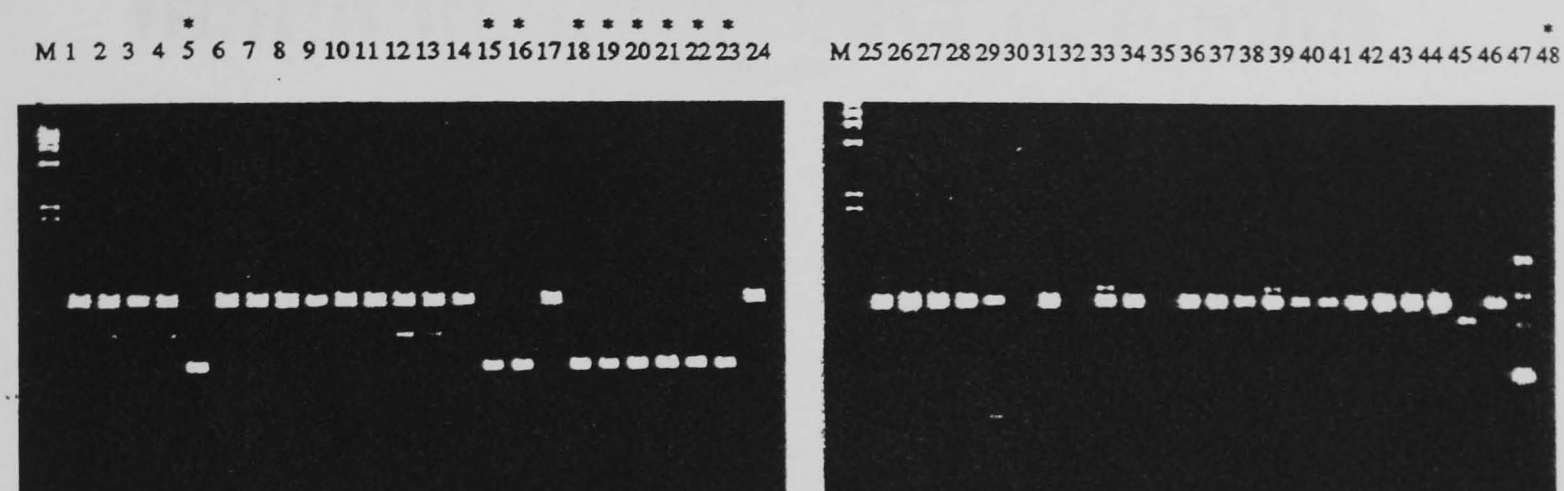
n.d. = not defined

Table 3.1.1 Isolates of *Microdochium nivale* from wheat listed according to tissue of origin, location, fungicide sensitivity, perithecial production and conidial and RAPD classification.

a) Primer: OPV08



b) Primer: OPY15



c) Primer: OPT14

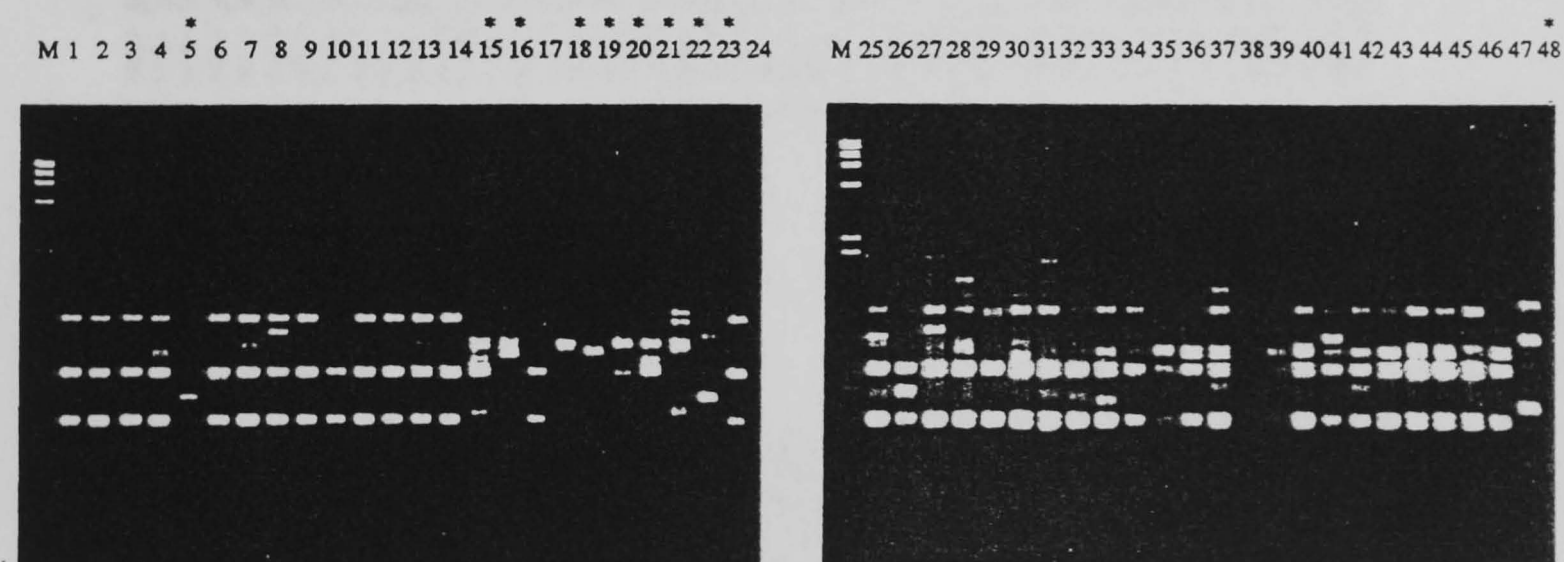
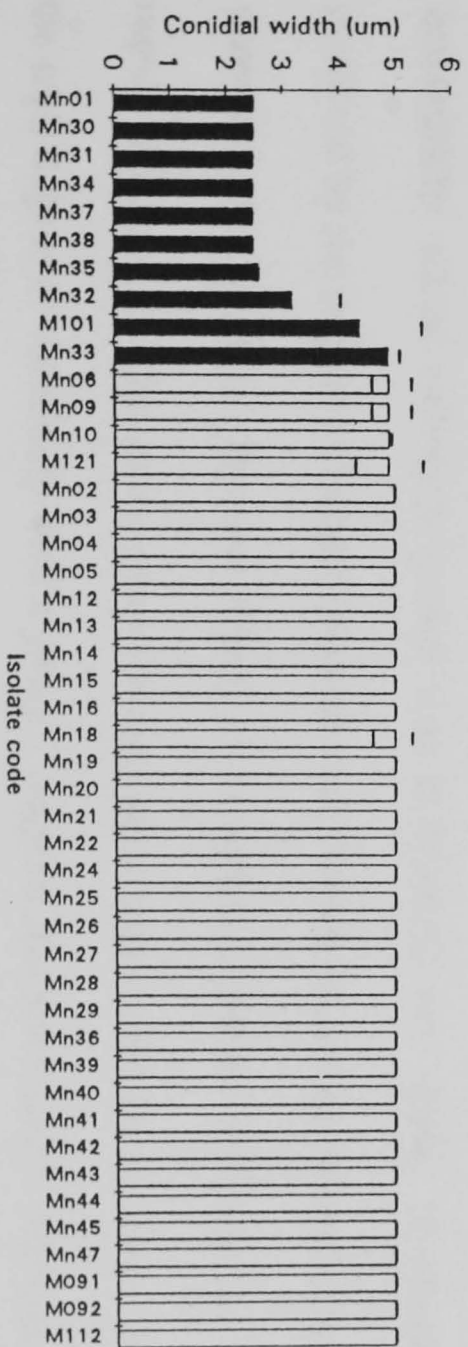
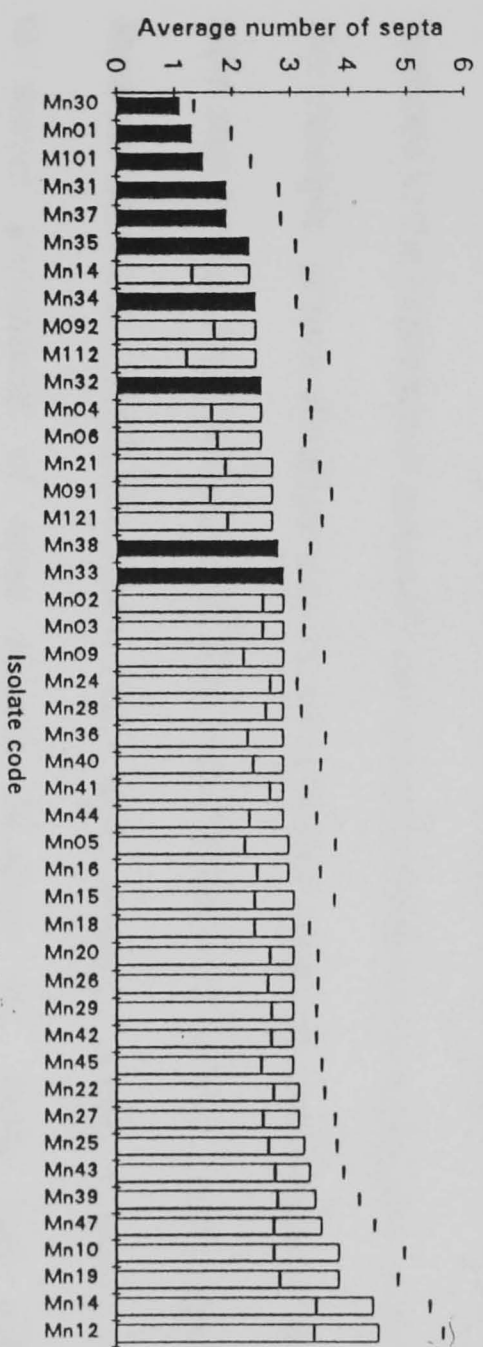
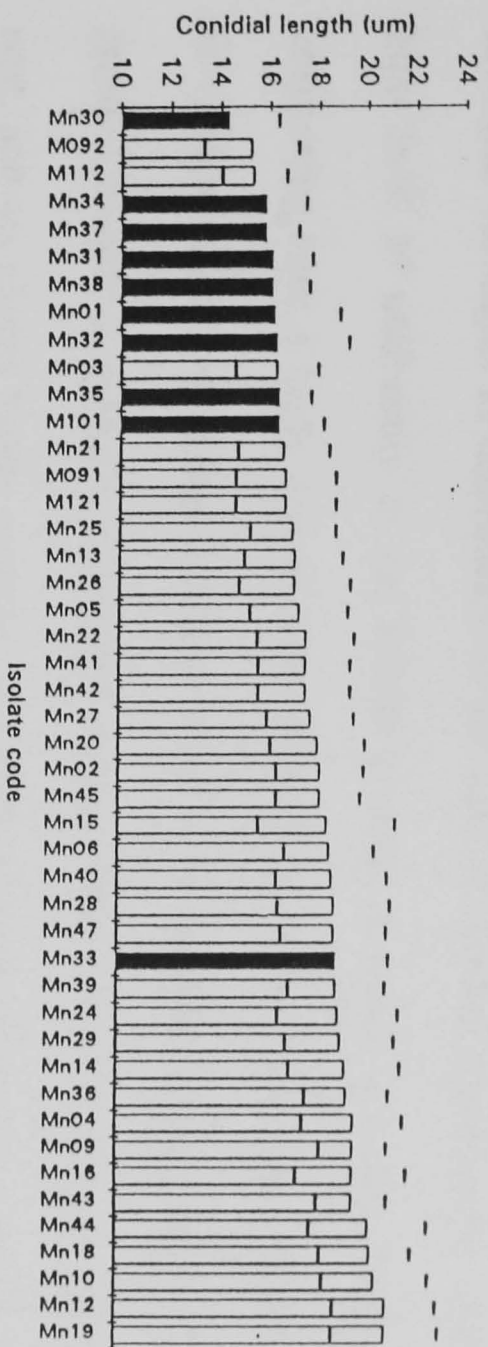


Fig 3.1.1. Random Amplified Polymorphic DNA profile of 48 isolates of *Microdochium nivale* produced by three primers OPV08 (a), OPY15 (b) and OPT14 (c). M= *hind*III cut lambda DNA with the sizes, from top to bottom, 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb. Lanes 1-48: M121, M112, M092, M091, M101, Mn48, Mn47, Mn45, Mn44, Mn43, Mn42, Mn41, Mn40, Mn39, Mn38, Mn37, Mn36, Mn35, Mn34, Mn33, Mn32, Mn31, Mn30, Mn29, Mn28, Mn27, Mn26, Mn25, Mn24, Mn22, Mn21, Mn20, Mn19, Mn18, Mn17, Mn16, Mn15, Mn14, Mn13, Mn12, Mn10, Mn09, Mn06, Mn05, Mn04, Mn03, Mn02, Mn01. DNA of isolate Mn03 amplified poorly relative to the other samples. DNA of isolates Mn17, Mn20 and Mn22 failed to amplify or produced very weak bands with primer OPY15 in Fig1b, but further samples from these isolates produced a profile similar to that of the other group 1 isolates. * denotes group 2 isolates.



Figs 3.1.2-3.1.4. Average conidial length (μm) and standard error of 46 isolates of *M. nivale* listed in table 3.1.1 (Fig 3.1.2). Average number of septa and standard error of the 46 isolates of *M. nivale* (Fig 3.1.2). Average conidial width (μm) and standard error of the 46 isolates of *M. nivale* (Fig 3.1.4). □ = RAPD Group 1 (*M. nivale* var. *majus*), ■ = RAPD Group 2 (*M. nivale* var. *nivale*).

DISCUSSION

RAPD profiles clearly distinguished a sub-group of *M.nivale* which broadly related to *M.nivale* var.*majus* as determined on the basis of conidial morphology. There was a high level of uniformity in the RAPD profiles of group 1 isolates, despite them originating from a fairly diverse range of sites. However, there was a greater degree of variation among the group 2 isolates (Fig 3.1.1). Examination of a larger number of group 2 isolates is needed to determine whether these group 2 isolates are of the same type, and are simply highly variable, or whether more than one type could exist within the group.

Low and high levels of variability between individuals of the same phenotype have been ascribed to the existence of asexually and sexually reproducing populations respectively. For example, genetic diversity was shown to be low in asexual populations of the wheat stem rust fungus *Puccinia graminis* f.sp. *tritici* (Burdon & Roelfs, 1985), and the sea anemone *Metridium senile* (Hoffman, 1986), and conversely, genetic diversity was high in sexual populations of these species. *M.nivale* var. *majus* may reproduce homothallically or by means of asexual conidia. If *M.nivale* var. *nivale* is indeed composed of a single group these preliminary results would imply that the extent of heterothallic sexual activity is greater than in *M.nivale* var. *majus*. Perithecia were produced by the majority of single spore derived isolates indicating that both groups are potentially homothallic. The low degree of variability observed within group 1 (var. *majus*) indicates that this group generally reproduce homothallically in nature, whereas the high degree of variability within group 2 suggests that heterothallic reproduction is more common in this group. Alternatively, Group 2 may constitute a number of types which also tend to reproduce by homothallic means.

All isolates were assigned to one of the two groups *M.nivale* var. *nivale* and *M.nivale* var. *majus* on the basis of their conidial morphology, as described by Gerlach &

Nirenberg (1982). The two types were distinguished by consideration of a combination of conidial characteristics. The two types were most easily distinguished by conidial width, with var.*nivale* being in general narrower than var.*majus* (Fig 3.1.4). The two types could not be distinguished on the basis of average number of septa or conidial length alone (1.1-4.6, 14.3-21.2 μ m respectively) as the data formed a continuum across the range of values (Figs 3.1.2-3). Type could therefore not be identified using any of these criteria alone.

Groups 1 and 2 identified by RAPDs were compared to the varieties distinguished by morphological characters and found to correlate to the varieties *M. nivale* var.*majus* and *M. nivale* var.*nivale* respectively. Thus the RAPD results support the evidence for the existence of two varieties as recorded by Gams & Muller (1980) and Gerlach & Nirenberg (1982). This is in contrast to the observations of Nelson *et al.* (1983), and Litschko & Burpee (1987) who could not differentiate isolates by any of the methods that they examined, and therefore suggested that distinct varieties of *M. nivale* did not exist.

One isolate (Mn33), identified by conidial morphology as var.*majus* clearly had a group 2 (var.*nivale*) RAPD profile. Conidial morphology is a continuous variable with overlap between the two types, even for conidial width, the most discriminatory of the conidial characteristics, whereas RAPDs provide a discrete grouping of isolates. This illustrates the capacity of the RAPD system to differentiate isolates of the var. *majus* subgroup from other *M. nivale* isolates.

In this study, the majority of isolates were classified as group 1 (var.*majus*). All isolates originating from grain, and a large proportion of those from the stem base were of the group 1 (var.*majus*) type. The absence of group 2 (var.*nivale*) isolates from grain is of interest, as it indicates that there may be a correlation between host tissue origin and isolate type. However, due to the small number of isolates obtained from grain, no firm

conclusions can be drawn.

The geographical and host tissue distribution of var.*nivale* and var.*majus* are not known, but the production of perithecia by both types *in vitro* raises the possibility that ascospore release may be an important factor in their epidemiology. Pettitt *et al* (unpublished) considered both types of *M.nivale* in their national disease survey, and found that both var.*nivale* and var.*majus* types were abundant in natural populations. In work carried out by Smith (1981) and Harris (1986), no perithecia were noted on isolates of *M.nivale* (var.*nivale*) obtained from turfgrasses. In this study, homothallic isolates conforming to *M.nivale* var.*nivale* were obtained from wheat. It would be interesting to examine isolates from turfgrass and to compare them with isolates from wheat to assess whether isolates from the two hosts could be considered to constitute a single population.

A difference in fungicide sensitivity of the two groups would have had implications in the control of the stem base disease complex of wheat. The balance of species present, and therefore the extent to which damage to the host occurs, could be influenced by selective control of one type. However, in the case of the MBC fungicide benomyl, the two groups showed no difference in their response and all but one isolate (Mn30, group 2) was resistant. The response of the two groups of *M.nivale* to other types of fungicide may however be different. The occurrence of the distinct sub-group of *M.nivale*, relating to var. *majus*, revealed by this work, raises the question of whether it occupies a different ecological niche or differs in pathogenicity, epidemiology or geographical and host distribution to other isolates of *M. nivale*. As *M.nivale* can now be considered to consist of at least two different varieties, it would be beneficial to derive probes for the identification of both of these forms.

4.0 Development and use of species specific primers for the identification of *Fusarium culmorum*, *Fusarium avenaceum* and *Microdochium nivale* as stem base pathogens of wheat.

Identification of the *Fusarium* and *Microdochium* species which cause stem base disease of wheat is not possible from examination of disease symptoms alone, and relies on isolation of the pathogens from plant material, and axenic culture techniques followed by identification of the causal organisms.

Fungal pathogens other than *Fusarium* or *Microdochium* which occur in the stem base of wheat can make selective isolation of these species difficult. The use of selective media for the isolation of *Fusarium* species has been documented by Nash & Snyder (1962), Tio *et al.* (1977) and Van Wyk *et al.* (1986). McMullen & Stack (1983) showed that the use of conventional media for the isolation of stem base pathogens can select for one particular species and Pettitt *et al.* (1993) described a method for the improved estimation of the incidence of *Microdochium nivale* in winter wheat by the use of a fungicide amended medium.

The systems previously used for the classification of *Fusarium* species vary. For example, Wollenweber (1931) divided the genus *Fusarium* into 16 sections which were themselves split into species, varieties and forms. In order to simplify this system, Snyder & Hansen (1940) described a species concept in which they changed the sections according to Wollenweber into individual species each having a number of biological forms. Later, Nirenberg (1981) described a method for identifying *Fusarium* species on wheat which took into consideration both the Wollenweber and Snyder & Hansen systems. In addition, methods for the identification of *Fusarium* species and *Microdochium nivale* are described by Booth (1971), Gerlach & Nirenberg (1982) and Brayford (1989).

From the literature, it is clear that classical taxonomy of *Fusarium* species is fraught with problems. Snyder & Hansen (1940) found difficulties in the identification of *Fusarium* species due to the capacity of isolates to vary widely in their morphological and physiological characteristics, and according to Brayford (1989) it is rarely possible to reliably identify *Fusaria* to species level directly from isolation plates since colonial and spore morphologies are usually atypical. For identification at species level isolations should be purified by sub-culturing, and for critical work, isolation of single spores is necessary.

After a few weeks in culture, the macroconidia produced by the isolate must be examined microscopically, and scored for a number of different morphological characteristics including conidial length, width and number of septa. Such identification criteria may overlap between species and lead to inaccurate identification of isolates.

A number of systems have been exploited to aid identification of fungal species and many of these may be applicable to the differentiation of *Fusarium* species. The ability of *Fusarium* and *Microdochium* species to produce mycotoxins has been exploited as an aid to taxonomy, as their production varies between species. Lauren *et al.* (1992) studied the production of trichothecenes by *Fusarium* species in order to determine the relationship between toxin types and species. They found that isolates of *F.culmorum* and *F.crookwellense* were mainly producers of the tricothecene nivalenol, and isolates of *F.graminearum* could be either deoxynivalenol or nivalenol producing chemotypes. Marasas *et al.* (1984) classified isolates of *M.nivale* on the basis of the absence of trichothecene production.

Additionally, isozymes have been used to differentiate species, and to examine variability within fungal populations. Isozyme variation has been used to determine the taxonomic status of organisms when the morphological characteristics were not distinctive. For example, Bosland & Williams (1987) characterised isolates of *Fusarium*

oxysporum by pathogenicity, isozyme polymorphism and vegetative compatibility. Erselius & Shaw (1982) used protein and enzyme differences between *Phytophthora palmivora* and *P.megakarya* to verify previously described taxonomic divisions. However, the use of isoenzymes in this way does have limitations. Each isozyme requires a unique assay which can be limited by the quantity of living material required for enzyme extraction, identification of new isozyme polymorphisms is not routine, and such polymorphisms may be infrequent in some pathogen populations, producing a limited number of useful enzyme systems.

The systems described above are based upon phenotypic characteristics, e.g. colony morphology, spore dimension, production of secondary metabolites. Such phenotypic characteristics are influenced by environmental effects on genotype, and therefore fluctuations in environmental conditions may affect the accuracy of such identification systems.

A direct approach for assessing genetic variation in fungi is by comparative study of molecular markers as described by Michelmore & Hulbert (1987). However, the most commonly used technique is the analysis of Restriction Fragment Length Polymorphisms (RFLPs). These result from specific differences in DNA sequence that alter fragment sizes obtained by digestion with a restriction endonuclease. RFLP analysis of mitochondrial and ribosomal DNA has been used to examine the relatedness of strains of *Fusarium oxysporum* (Kistler *et al.*, 1987), to relate vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f.sp.*dianthi* (Manicom, 1990), and to analyse *Fusarium* species from cereals (Nicholson *et al.*, 1993). RFLPs have advantages as molecular markers as the number of RFLP markers is effectively unlimited, and if a sufficient number of markers are identified, they can be used to develop detailed genetic maps which are useful tools for the study of variation, and for cloning genes of interest. For example, genetic linkage maps of lettuce (*Lactuca sativa*),

and the fungus causing lettuce downy mildew (*Bremia lactucae*) were constructed using RFLP markers (Landry *et al.*, 1987, Hulbert *et al.*, 1988).

Kistler (1991) used DNA probes to determine relatedness between strains of *Fusarium oxysporum*, and Henson (1989), cloned a mitochondrial DNA fragment from *Gaumannomyces graminis* which showed little homology with DNA from other fungi, and was therefore used in the identification of the pathogen. Similarly, Yao *et al.* (1991) cloned DNA fragments to produce probes for the identification of *Peronosclerospora sacchari*, and Koopmann *et al.* (1994) produced species-specific probes that were able to differentiate between *F.culmorum* and *F.graminearum*.

The use of techniques such as RFLP has allowed the structure and variation within fungal populations to be examined. However, RFLP analysis is time consuming and laborious and generally requires a DNA library for the preparation of probes.

Using the polymerase chain reaction (PCR) it is possible to amplify specific regions of the genome. The polymerase chain reaction can selectively amplify a particular stretch of template DNA exponentially, and involves the enzymatic amplification of a DNA fragment flanked by 2 oligonucleotide primers hybridizing to opposite strands of the target sequence. The Random Amplified Polymorphic DNA (RAPD) assay as described by Williams *et al.* (1990) involves the amplification of genomic DNA by PCR using single primers of arbitrary nucleotide sequence, and provides a simple method for the detection of polymorphisms in the absence of specific nucleotide information. RAPD markers are fragments of DNA to which 10 base oligonucleotide primers have annealed at either end, and have been amplified exponentially by PCR. Polymorphisms between species, pathotypes and isolates can be identified directly following electrophoretic size fractionation of RAPD products in agarose gels. Correll *et al.* (1993) showed that there was a strict correspondence between RFLP haplotype and RAPD grouping in isolates of *Colletotrichum orbiculare*. The use of RAPDs in the identification and differentiation

of fungal pathogens is well documented. For example, Goodwin & Annis (1991) were able to discriminate virulent and avirulent isolates of *Leptosphaeria maculans* and Grajal-Martin *et al.* (1993) characterised races of *F.oxysporum* using the RAPD assay. Nicholson & Rezanoor (1994) used RAPDs to identify pathotype and detect variation in *P.herpotrichoides*, and Guthrie *et al.* (1992) were able to identify and differentiate isolates of *Colletotrichum graminicola*.

Disease diagnosis at present relies on observation of symptoms. Early in the season, stem base disease symptoms caused by *Fusarium* species and *M.nivale* cannot be discriminated reliably from those of eyespot and sharp eyespot. Therefore, reliable disease assessments cannot be made early in the season and decisions to apply fungicides may be too late to control the disease effectively, even if a systemic fungicide is applied. The use of more reliable techniques for the identification of *Fusarium* and *Microdochium* species would be helpful for the rapid diagnosis of foot rot disease, and pre-symptomatic identification would allow more timely decisions concerning fungicide applications. A number of systems for the rapid identification of stem base pathogens have been developed.

All the procedures described so far to identify fungal pathogens have required isolation of the fungus from the plant. Other techniques have been used for the detection of the pathogens in plant material.

Polyclonal and monoclonal antibodies have been used widely in the detection of fungal species. Höxter *et al.* (1991) developed an indirect enzyme linked immunosorbent (ELISA) assay, and were able to detect infection by *Microdochium nivale* in rye using polyclonal antibodies, and Lind (1990) described the isolation of antigens for the serological identification of *Pseudocercospora herpotrichoides*, and subsequently, the application of polyclonal antibodies directed against these proteins to show a correlation with disease symptoms (Lind, 1992). Unger & Wolf (1988) documented the use of

polyclonal antibodies for the detection of *P.herpotrichoides* in wheat, but found that there was some cross reaction with other species. These workers also failed to test the sharp eyespot pathogen *Rhizoctonia solani*, which may also have cross-reacted with the polyclonal antibodies.

Monoclonal antibody (Mab) assays, which involve the use of only one antibody, as opposed to the range of antibodies used in polyclonal assays, are thought to produce less cross-reactivity with other species than polyclonal antibodies (Dewey *et al.*, 1994). The detection of *P.herpotrichoides* (Dewey *et al.*, 1994) using monoclonal antibodies has been described, although cross-reaction between *P.anguoides* and *P.herpotrichoides* can still be a problem. The difficulties in raising specific antisera to fungi that do not cross-react with related species, species from unrelated genera, and host molecules is discussed by Dewey (1988). Neither polyclonal nor monoclonal antibodies produced for the detection of *P.herpotrichoides* are able to differentiate between the different pathotypes of this pathogen. The detection of fungi at low levels using Mab assay may be unreliable, and it is therefore questionable whether such assays are always sensitive enough for diagnostic purposes. In addition, Dewey (1988) found that fungal antisera cross-react with unrelated species even at high concentrations. Thus in many cases, serological means have failed to produce the desired species and subspecies specificities. Nicholson *et al.* (1994) identified a pathotype-specific DNA probe for the R-type of *P.herpotrichoides*, and demonstrated its ability to detect this pathogen in infected rye seedlings, and Henson (1989) cloned a mitochondrial DNA fragment, and used it as a specific probe for the detection of *Gaumannomyces graminis* in infected plant material. Bereswill *et al.* (1992) cloned and sequenced a species-specific DNA fragment from *Erwinia amylovora*, and produced oligonucleotides for the sensitive and specific detection of the pathogen in apple seedlings. Similarly, Seal *et al.* (1992) isolated a *Pseudomonas solanacearum* specific DNA fragment from which they produced species-

specific primers for the detection of low numbers of the bacteria, by PCR amplification and Bej *et al.* (1991) amplified regions of a gene coding for B-glucuronidase by PCR, and developed a PCR amplification-gene probe detection method for detecting *E.coli*. Random species-specific DNA sequences identified using the RAPD assay are useful only for the identification of pure cultures, as DNA from the host or other fungal species would produce additional amplification products. However, such sequences can be further developed to produce species-specific oligonucleotide primers for use with PCR, and therefore for the detection of the pathogen in the host tissue. This approach has been used by Ouellet & Seifert (1993) who characterised strains of *F.graminearum* using a RAPD assay, and subsequently designed specific primers for the detection of these strains. Schafer & Wostmeyer (1994) also used a RAPD-PCR system to develop species-specific identification of *Leptosphaeria maculans*.

In the present study, RAPD assays were used for the identification of species specific PCR amplification products in *F.culmorum*, *F.avenaceum* and the two types of *M.nivale*. Selected RAPD products were cloned, and the clones were analysed and hybridised to Southern blots of genomic DNA from a range of stem base pathogens to identify clones which exhibited specificity for the species of origin. The selected clones were sequenced and analysed to produce primer pairs for use in PCR. The primer pairs for each pathogen were evaluated to determine their ability to identify and detect the pathogens *in vitro* and in plant tissue.

4.1 Random Amplified Polymorphic DNA (RAPD) analysis and development of species specific oligonucleotide primers for use in PCR.

MATERIALS AND METHODS

Origin and maintenance of fungal isolates

Twenty four isolates of each of the species *F.culmorum* and *F.avenaceum* and eight isolates of *M.nivale* (two var.*nivale*, and six var.*majus*) were isolated from stem bases of wheat originating from several sites at Harper Adams Agricultural College. Cultures were maintained on Potato Dextrose Agar (PDA) (Difco) plates containing streptomycin sulphate (100µg ml⁻¹), neomycin (50µg ml⁻¹) and chloramphenicol (50µg ml⁻¹) at 15°C.

DNA extraction and RAPD analysis.

Mycelium was scraped from the surface of the fungal colonies and added to 500µl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA) in a 1.5 ml Eppendorf tube. The tube was placed in boiling water for 5 min., removed and placed on ice. Tubes were then centrifuged for 10 min at 13000 r.p.m.

The DNA from the 24 isolates of *F.culmorum* or *F.avenaceum* was bulked together for the initial screening of primers. Primer kits OPA, OPT, OPU, OPV, OPW, OPX, and OPY (Operon Technologies Inc. Alameda CA94501, US) each consisting of 20, 10-mer primers were screened against *F.culmorum* and *F.avenaceum* bulk DNA mixtures. The *F.culmorum* and *F.avenaceum* mixtures were screened in tandem with each of the primers in order to distinguish products present in only one of the species. *Microdochium nivale* (var.*nivale* and var.*majus*) isolates were screened using primer kits OPT, OPV and OPY.

Amplification reactions were performed in volumes of 50µl containing 10ng of genomic template DNA. The reaction buffer consisted of 100µM each of dATP, dCTP, dGTP

and dTTP, 200nM oligonucleotide primer, and 0.8 units of *Taq* polymerase (Boehringer Mannheim Ltd), in 10mM Tris: HCl (pH8.3), 1.5mM-MgCl₂, 50mM-KCl, 100µg ml⁻¹ gelatine, 0.05% Tween 20, and 0.05% Nonidet P-40. Reaction mixtures were overlaid with mineral oil prior to PCR.

Amplification was performed in a Perkin Elmer Cetus DNA thermal cycler programmed for 45 cycles of 1 min at 90⁰C, 1 min at 36⁰ and 2 min at 72⁰ with transition times of 114s between denaturing and annealing, and 72s between annealing and extension. Aliquots (10µl) of amplification products were fractionated by electrophoresis through 1.5% agarose gels and detected by staining with ethidium bromide.

Those primers which produced bright, distinct bands when amplified with either *F.culmorum* or *F.avenaceum*, but not both were selected and retested. Primers OPU08, OPU11, OPU17, OPU19 and OPW19 were retested against the 10 separate isolates of *F.culmorum* and DNA from a selection of other stem base pathogens including *F.graminearum* which is closely related to *F.culmorum*, and was therefore thought likely to show cross reaction with this species. Likewise, primers OPU17, OPW03, OPW19 and OPY07 were retested with the separate *F.avenaceum* isolates.

Primers OPV08, OPT14, OPY13, OPY14, and OPY15 were selected for further investigation of the 8 *Microdochium nivale* isolates. (For primer sequence information see appendix 4.1.1)

Cloning of the PCR product

PCR products obtained by the amplification of fungal DNA with the selected primers (*F.culmorum* = OPU08, OPU11, OPU19, OPW19, OPU17, *F.avenaceum* = OPW03, OPW19, OPU17, OPY07, and *M.nivale* = OPY13, OPY14, OPY15) were

electrophoresed through a 2% low melting point gel and were compared to those of previous RAPD assays using the same primers to ensure that the bands were of the same size. The selected bands were excised under U.V. light. The DNA from these bands was purified using the Magic™ PCR Preps DNA Purification System (Promega, Madison WI 53711 U.S.A) according to the manufacturers instructions. The DNA was purified to ensure that the preferential incorporation of lower molecular weight PCR products, or incorporation of extraneous product did not reduce the final number of colonies containing the relevant insert. The DNA recovery was checked by electrophoresis of 5µl aliquots of the purified samples through a 1.5% agarose gel. From the gel, the size (Kb) of the product to be used as an insert was estimated by comparison with a 2kb lambda HindIII marker. The amount of each PCR product needed to obtain the 1:1 molar ratio with the PGEM-T Vector optimal for ligation was calculated. The pGEM-T Vector System (Promega, Madison WI 53711 U.S.A) was used for the cloning of PCR products according to the manufacturers instructions. This vector has a 3' terminal thymine which overhangs the insertion site at each end and improves the efficiency of ligation of the PCR product into the plasmid. Ligation using these overhangs takes advantage of the non-template dependent addition of a single deoxyadenosine to the 3' end of PCR products by thermostable polymerases. Ligation reactions were set up to contain 1µl T4 DNA Ligase Buffer, 1µl PGEM-T Vector (50ng), the calculated amount of PCR product to give a 1:1 molar ratio (vector:insert), 1µl T4 DNA Ligase and H₂O to a final volume of 10µl. To transform the ligated PCR:pGEM-T vector, four microlitres of the reaction mixture was added to 40µl of a suspension of electro-competent *Escherichia coli* cells (strain JS 5, Bio-Rad Laboratories, CA94547 U.S.A.) in a cold 1.5 ml Eppendorf tube and left on ice for 1 minute. The mixture was transferred to a cold 0.1cm electroporation cuvette and a pulse of 1.8KV applied using a high voltage electroporation device (*E.coli* Pulser, Bio-Rad).

Immediately after electroporation, 1ml of SOC medium (Sambrook *et al.*, 1989) was added to the suspension, and the cells resuspended. The cell suspension was incubated at 37°C for 1 hour, after which selection for transformants was carried out by plating a 100 μ l aliquot of cell suspension onto LB/carbenicillin/IPTG/X-GAL indicator plates (Sambrook *et al.*, 1989). The plates were incubated overnight at 37°C. Insertional inactivation of the β -galactosidase coding region of the vector allows the recombinant clones to be directly identified by colour screening on indicator plates. The plates were examined, and four white (recombinant) colonies were removed from each sample and added to separate tubes containing 5ml of Luria-Bertani (LB) broth (Sambrook *et al.*, 1989). The tubes were incubated overnight at 37°C and 1-3ml of cells pelleted by centrifugation. The plasmid DNA was then purified using The Magic Minipreps DNA purification system (Promega) according to the manufacturers instructions. The purified miniprep DNA was diluted 100 fold with TE buffer and 3 μ l of this DNA was amplified in the presence of M13 forward and reverse universal primers in a reaction buffer consisting of 100 μ M each of dATP, dCTP, dGTP and dTTP, 0.8 units of *taq* polymerase (Boehringer Mannheim Ltd), in 10mM Tris: HCl (pH8.3), 1.5mM-MgCl₂, 50mM-KCl, 100 μ g ml⁻¹ gelatine, 0.05 % Tween 20 and 0.05 % Nonidet P-40. Reaction mixtures were overlaid with mineral oil prior to PCR. Amplification was performed in a Perkin Elmer Cetus DNA thermocycler programmed for 32 cycles of 1 min at 94°C, 1 min at 40°C and 2 min at 70°C using the fastest possible transition times. The M13 primers enable the insert to be amplified in a PCR reaction, as they correspond to sequences in the vector, on either side of the insert region. A 10 μ l aliquot of the PCR product was electrophoresed through a 1.5 % agarose gel, and the product bands examined to determine their size. The bands produced in the PCR reaction should be larger than the insert bands due to the M13 sites at the 5' and 3' ends of the insert occurring 62 and 45 bases away from the insert site respectively.

Southern blotting

Total DNA from *M.nivale* var.*nivale*, *M.nivale* var.*majus*, *Rhizoctonia cerealis*, *Gaumannomyces graminis* var.*tritici*, *P.herpotrichoides* a) W-type b) R-type c) C-type, *P.anguoides*, *F.culmorum*, *F.avenaceum*, *F.graminearum* and *F.poa* was digested with the restriction enzyme *Eco* R1 (Gibco Ltd) and electrophoresed through 0.8% agarose gels. Gels were stained with 0.5µg/ml ethidium bromide and the DNA was visualised on a UV transilluminator. DNA was transferred from gels onto nylon membranes (Hybond N+. Amersham International) by alkaline Southern blotting (Reed & Mann, 1985).

Preparation of probes and hybridization to membrane bound DNA.

Putative species specific clones were passed to P.Nicholson, and were then labelled with [³²P]dCTP (Amersham International, UK) to high specific activity by the 'oligo-labelling' method (Feinberg & Vogelstein, 1984) and hybridised onto filters of size fractionated *Eco*RI-digested total genomic DNA of a wide range of stem base pathogens. All membranes were prehybridized, hybridized and washed using standard procedures as described by Nicholson *et al.* (1993). Hybridization was detected by exposure to Kodak XAR-5 film at -70°C between two intensifying screens for 1-3 days. Clones were then passed to J.Smith and sequenced using an Auto Read Sequencing kit (Pharmacia) and analysed with an Automated Laser Fluorescent (ALF) DNA sequencer (Pharmacia). The sequence data were analysed using 'Primer' a programme for selection of primers for use in PCR.

Forward and reverse primers with a theoretical melting temperature of around 62°C were chosen for each species.

4.2 The use of species specific primers for the detection of *F.culmorum*, *F.avenaceum* and *M.nivale* in vitro.

In order to determine the ability of the primers developed to identify each of the pathogens, a range of isolates of the same species was amplified using the relevant primers. Twelve isolates of each of the pathogens *F.culmorum*, and *F.avenaceum*, and 24 isolates of *M.nivale*, 12 var.*nivale* and 12 var.*majus* type were amplified in the presence of their putative specific primers using the same PCR conditions previously described, but with an annealing temperature of 60°C. A range of stem base pathogens (*F.culmorum*, *F.avenaceum*, *F.graminearum*, *F.poa*, *M.nivale* var.*nivale*, *M.nivale* var. *majus*, W-type eyespot, R-type eyespot, and *Gaeumannomyces graminis*) was also tested with each of the putative species specific PCR primers under the same amplification conditions, to check for cross specificity with other species.

The range of detection of the species *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus*, firstly in the presence of only fungal DNA, and then within a plant DNA background was then assessed. Fungal DNA was extracted from each of the species using the method of O'Dell *et al.* (1989) and diluted to a concentration of approximately 120pg/μl with water. A serial dilution of this DNA was made and 1μl of DNA (concentration ranging from 4pg-60fg/μl) was amplified in a PCR reaction with the appropriate species specific primer. The minimum amount of DNA needed for amplification using the primers was determined, and the primers were also tested for their sensitivity in the presence of a background of plant DNA. Ten microlitre aliquots of plant and diluted fungal DNA were electrophoresed through a 0.8% agarose gel, and a comparison of their concentrations made according to intensity. From this gel, the concentration of plant DNA was estimated to be 4 times that of the diluted fungal DNA (500pg/μl), and a 100:1 dilution of the plant: fungal DNA was made. A dilution series ranging from 120pg/μl to 60fg/μl of fungal DNA was tested in the presence of the plant

DNA.

4.3 Use of the species specific primers *in vivo*.

1) Wheat seed (cultivar Mercia) was surface sterilised in sodium hypochlorite and washed in two changes of sterile distilled water. Seeds were imbibed overnight in darkness in sterile distilled water and planted in vermiculite in 10cm diameter pots with plastic propagator lids at a rate of 15 per pot. Seedlings were allowed to grow for two weeks at 15°C in a temperature controlled growth room under a 16 hr light/dark regime before inoculation. Seedlings were inoculated by placing 5mm plugs of agar from the edge of an actively growing colony of the pathogen next to the base of the plant. Pots were inoculated with one isolate of either *F.culmorum*, *F.avenaceum*, *M.nivale* var.*nivale* or *M.nivale* var.*majus*. After a further 4 weeks, 2cm sections from the base of ten plants were removed from each pot and bulked together.

It was decided that although species specific primers had been developed in order to make a tool capable of detecting all the foot rot pathogens, the *F.avenaceum* primer would not be used in the further studies involving inoculated plant material, as it was not considered to be a serious pathogen of the wheat stem base.

A test was conducted incorporating a fungicide application. Wheat seed (c.v. Mercia) was sown at a rate of 15 seeds/pot in John Innes number 2 potting compost in 4cm diameter pots in a controlled environment cabinet (conviron). Conditions were kept constant at a temperature of 10°C and 75% relative humidity. Pots were watered to a constant weight. After 2 weeks, the seedlings were inoculated as above. There were 16 treatments consisting of the eight combinations of inoculations of the 3 species *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus*, and an uninoculated control, (Treatments 1-8 corresponded to treatments 9-16). The 16 treatments had 4 replicates, arranged in blocks. Three weeks after inoculation, treatments 9-16 were removed from

the cabinet, and sprayed with a field application of the triazole fungicide flusilazole (250l/ha, 40% w.v a.i.). Pots were then returned to the cabinet for a further 7 weeks before harvest. Ten plants were sampled from each pot, and this sample then divided in two. There were therefore 2 samples from each pot, and 4 replicates giving 8 samples from each treatment.

A third test was conducted in which combinations of fungal inocula were used. In this test, plants were grown at a rate of 15 per pot as above, and 4 replicate blocks each containing 8 treatments were set up. Treatments consisted of inoculation of every combination of the species *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* and an uninoculated control. Seedlings were allowed to grow for two weeks at 15°C in a temperature controlled growth room under a 16 hr light/dark regime before inoculation, and were harvested 6 weeks following inoculation. Sections 2cm from the stem bases of six separate plants were harvested from each pot, and the remaining plants from each pot were bulked together. 32 samples of single plants and 4 bulk samples were obtained from each treatment.

DNA was extracted from each of the plant samples collected in the previous tests as follows. Samples were freeze-dried and stored at -20°C in pre-weighed plastic tubes. The weight of the freeze dried plant material was calculated so that final DNA dilutions could be made according to the amount of plant tissue originally sampled. Each sample was then ground in a mill (Glen Creston Ltd, Stanmore; mixer/mill 8000) for 6 minutes, transferred to a 15ml tube and 7 ml of CTAB (hexacetyltrimethylammonium bromide) buffer (CTAB 8g, sarkosyl 10g, sorbitol 25g, NaCl 47g EDTA 8g, polyvinylpolypyrrolidone (PVPP) 10g in 1l H₂O) and 50µl of 10mg/ml proteinase k were added. Tubes were incubated at 65°C for 2 hours and shaken occasionally, after which 7ml of chloroform was added to each tube. Tubes were shaken, centrifuged for 10 min at 3000r.p.m., and the upper aqueous phase removed to a new tube containing 6ml of

ice cold isopropanol. Tubes were left at -20°C for at least 30 mins, centrifuged for 10 min at 3000 r.p.m., and the supernatant discarded. The remaining pellet was washed with 1ml of 70% ethanol and allowed to dry before being dissolved in tris/EDTA (10mM tris-HCl, pH 8.0, 0.1mM EDTA) buffer. The amount of TE buffer (5 μ l/mg of dry weight tissue) used to dissolve the samples was calculated relative to the initial weight of the dry plant material. A 1 μ l aliquot of this extracted DNA was amplified in the presence of one of the species specific primers. Each of the samples was amplified in the presence of the *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* primers. Amplification was carried out using 40 cycles of 30 sec at 95°, 20 secs at 60° and 45 secs at 72° using the fastest possible transition times. A 15 μ l aliquot of the PCR product was electrophoresed through a 1.5% gel, the DNA was stained with ethidium bromide and visualised using a U.V. transilluminator. The absence or presence of each species in each sample was noted, and each sample was scored on a scale of 0-3 for the intensity of the band produced, 0 = no band, 1 = faint band, 2 = moderate band, 3 = bright band.

RESULTS

Identification and cloning of RAPD fragments from *F.culmorum*, *F.avenaceum* and *M.nivale* (var.*nivale* and var.*majus*).

Several of the Operon primers tested produced bright distinct bands when amplified with the *F.avenaceum* and *F.culmorum* bulk DNA samples. Those which showed good amplification of one of these species compared to the other were selected as the most likely to be specific to that particular species. When tested with a range of isolates of the same species in order to determine their reliability, the primers selected for *F.culmorum* (OPU08, OPU11, OPU19, OPW19, OPU17) and *F.avenaceum* (OPW03, OPW19, OPU17, OPY07) produced good amplification and there was no variation between isolates. Isolates of *F.culmorum* and *F.avenaceum* amplified using these primers are shown in Figs 4.1.1 and 4.1.2 respectively. These primers were therefore selected for further development. Primer U17 showed a clear differentiation between isolates of *F.culmorum* and an isolate of *F.graminearum* tested with the same primer (Fig 4.1.3). Those bands indicated with white arrows in Fig 4.1.1-3 show the products selected for cloning. In some cases more than one band was chosen from each primer. When isolates of *Microdochium nivale* were amplified in the presence of the primers selected (OPY13, OPY14, OPY15) two distinct banding patterns were observed with primers OPY13 and OPY15 (Fig 4.1.4). One form occurred in isolates number 1 and 6 (the var.*nivale* type), while a second form occurred in the remaining isolates (var.*majus*). The primer OPY13 produced a major amplification product of approximately 0.8kb with var.*majus* type isolates, compared to a 0.7kb product with the var.*nivale* type isolates. Similarly primer OPY15 produced highly amplified fragments of approximately 1.2kb and 0.5kb with the var.*majus* and var.*nivale* types respectively. The OPY14 primer did not differentiate between the two types of *M.nivale*, and produced a product of 1.1kb in all isolates. The white arrows in Fig

4.1.4 indicate the products chosen to be selective for *M.nivale* var. *nivale* and *M.nivale* var. *majus* (OPY13, OPY15) and for *M.nivale* as a species (OPY14). The selected fragments were isolated from the remaining amplification products as described above, and are shown in Fig 4.1.5. The products were then cloned.

Amplified insert DNA from the recombinant colonies and a sample of the original PCR product were run on an 0.8% agarose gel. Those clones where the amplified inserts were of the same size (ie showed reproducibility), but were slightly larger than the original PCR product amplified with the operon primer due to a slight extension caused by their amplification with the M13 primers were chosen for hybridisation.

Hybridisation of RAPD fragments from *F.culmorum*, *F.avenaceum* and *M.nivale* (var.*nivale* and var.*majus*) to DNA from a range of stem base pathogens.

The profiles shown by the hybridisation of the RAPD fragments from *F.avenaceum* (U17A2, Y07A, W03A), *F.culmorum* (U08C, U171C, U172C) and *M.nivale* (Y13, Y14, Y15) to the Southern membranes of DNA from 12 stem base pathogens were examined, and the cloned fragments assessed for cross reaction between species or varieties as relevant.

In the case of the *F.avenaceum* specific fragments (Fig 4.1.6), the fragment U17A2 showed good affinity to the *F.avenaceum* DNA, producing a band approximately 4.3Kb in size, but not to the other *Fusarium* species. Fragment Y07A hybridised to the *F.avenaceum* DNA (4Kb and 8.5Kb), but also showed cross hybridisation to *F.culmorum* (2Kb), *F.graminearum* (9.5Kb) and *F.poa* (9.2Kb), whereas fragment W03A did not hybridise to the *F.avenaceum* DNA, but showed some hybridisation to the *F.poa* DNA, producing a band of approximately 8Kb.

Similarly for the *F.culmorum* specific fragments (Fig 4.1.7), U08C showed good hybridisation to the *F.culmorum* DNA (2.2Kb), a lower intensity band of a different

size (9.5Kb) with the *F.graminearum* and no hybridisation to *F.poa*. U171C and U172C hybridised to *F.culmorum*, *F.graminearum* and *F.poa*. *F.poa* produced bands of a different size to the other two species using both fragments, and could therefore be differentiated on the basis of RFLP, but there was no differentiation between *F.culmorum* and *F.graminearum* with either of U17C fragments.

In the case of the fragments produced for the *M.nivale* isolates (Fig 4.1.8), fragment Y14 hybridised to both the *nivale* and *majus* DNA, and produced bands of approximately 8Kb. The Y13 *majus* and *nivale* fragments hybridised to the *majus* (2kb) and *nivale* (9.4Kb) DNA respectively, and showed only limited hybridisation to the DNA of the alternative variety. The Y15 *majus* fragment showed a clear band with only the *majus* DNA and very little hybridisation to the *nivale* DNA, but the Y15 *nivale* fragment also showed some cross hybridisation to the *majus* DNA.

Amplification of *F.culmorum*, *F.avenaceum* and *M.nivale* (var.*nivale* and var.*majus*) fungal DNA using putative species specific primers

When the putative species specific primers for *F.culmorum*, *F.avenaceum* and *M.nivale* (var. *nivale* and var.*majus*) were used to amplify several isolates of each pathogen, all isolates of each type could be detected, and there was no polymorphism between isolates of the same species (Fig 4.2.1-4.2.2). There was also no amplification of any other stem base pathogens using these specific primers. Fig 4.2.3 shows the PCR amplification of a range of stem base pathogens (*Rhizoctonia cerealis*, *P.herpotrichoides* (W and R-type), *F.graminearum*, *F.poa*, *F.culmorum*, *F.avenaceum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus*) using the *M.nivale* var.*nivale* and *M.nivale* var.*majus* specific primers, where only the *M.nivale* var.*nivale* and var.*majus* DNA has been amplified. Similar results were obtained using the putative *F.culmorum* and *F.avenaceum* primers, with only DNA from those pathogens being amplified in each

case. The amplification of dilutions of fungal DNA with the primers developed, showed that the primers were efficient at detecting each of the 3 fungal pathogens at concentrations of greater than 1pg/ μ l DNA *in vitro* (Fig 4.2.4). The addition of a plant DNA background to the reaction did not affect amplification (Fig 4.2.5).

The use of species specific primers to detect *F.culmorum*, *F.avenaceum* and *M.nivale* (var.*nivale* and var.*majus*) in plant material.

In a preliminary test, where individual seedlings were inoculated with either *F.culmorum*, *F.avenaceum*, *M.nivale* var.*nivale* or *M.nivale* var.*majus*, the species specific primers were able to detect each of these pathogens in plant tissue using PCR. For example, Fig 4.3.1 shows the amplification products produced by the species specific *F.culmorum*, *F.avenaceum* and *M.nivale* var.*nivale* and var.*majus* primers with both pure fungal and DNA extracted from inoculated plants.

The fungicide tests (treatments 9-16) conducted at 10°C showed a high level of cross contamination between treatments particularly by *F.culmorum* and *M.nivale* var.*majus* (Table 4.3.1) The fungicide application did not reduce the number of samples infected with any of the 3 pathogens. It was noted however that even though a very high proportion of samples were infected, those which had been artificially inoculated produced more intense amplification products.

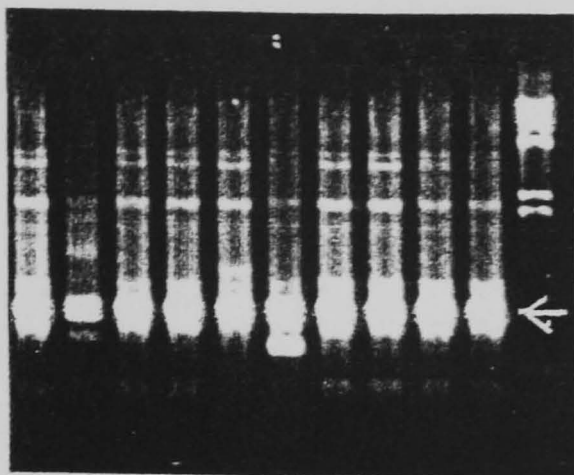
The co-inoculation experiment conducted at 15°C showed good amplification of *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* with the primers tested. This is illustrated in Fig 4.3.2 which shows the amplification of one block of treatments by each of the 3 primers. There was a low level of amplification of all three pathogens in several of the uninoculated plants. However, the predominant bands in each of the treatments matched the species with which the plant had been inoculated. These bands tended to be of a much higher intensity than those produced by the background DNA,

and indicated that significant infection of the inoculated species had taken place. An example of this is shown in Fig 4.3.2, which illustrates the difference in band intensity using primers specific for *F.culmorum* in a block of plants some of which had been artificially inoculated with *F.culmorum*, and the remainder which were apparently naturally infected or contaminated.

Table 4.3.2 shows the number of plants from each treatment infected with each pathogen, and each combination of pathogens according to treatment. Over all treatments 21 plants (11%) not inoculated with *F.culmorum* showed the presence of the fungus, and this was similar for var.*nivale* with 26 (13.5%) plants infected. There was a lower level of infection of var.*majus*, with 6 (3.2%) plants infected. In those treatments inoculated singly with *F.culmorum*, var.*majus* or var.*nivale*, the samples tended to show high levels of those fungi alone, as would be expected. In treatments 2-7, there was a high occurrence of *F.culmorum* in conjunction with the inoculated species in each treatment. For example in treatment 2, where plants had been inoculated with *M.nivale* var.*majus* alone, five plants showed a dual var.*majus* and *F.culmorum* infection. When species were co-inoculated, there was a tendency for both species to be detected within one plant, rather than plants being infected exclusively by one species. The control treatment (Treatment 8) had 13 contaminated plants, of which the predominant infecting species were *F.culmorum* and *M.nivale* var.*nivale*. When the bands were scored for their intensity (Table 4.3.3), DNA from plants in those treatments which had been artificially inoculated produced high intensity bands when amplified with the relevant primers compared to those bands produced by DNA from the non-inoculated, or naturally infected plants amplified with the same primers. For example, 12-15 plants in each of the treatments inoculated with *F.culmorum* alone, or in combination with other species (treatments 1, 4, 5, 7) had the highest brightness intensity score (3), compared to a score of 0 in all of the other treatments (Table 4.3.3).

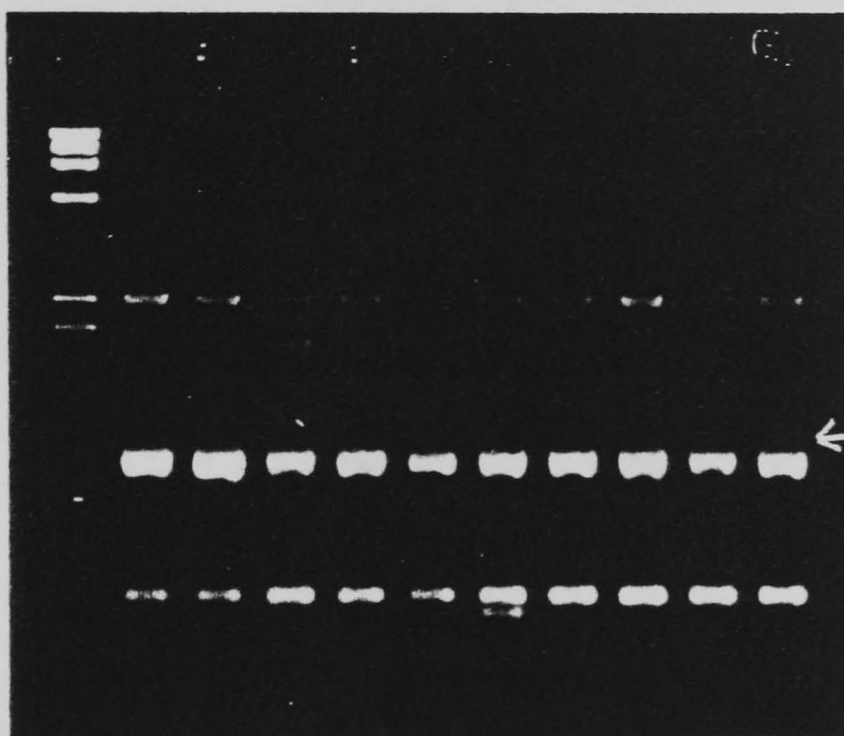
1 2 3 4 5 6 7 8 9 10 λ

Primer: OPU08



λ 1 2 3 4 5 6 7 8 9 10

Primer: OPU11



λ 1 2 3 4 5 6 7 8 9 10

Primer: OPU17

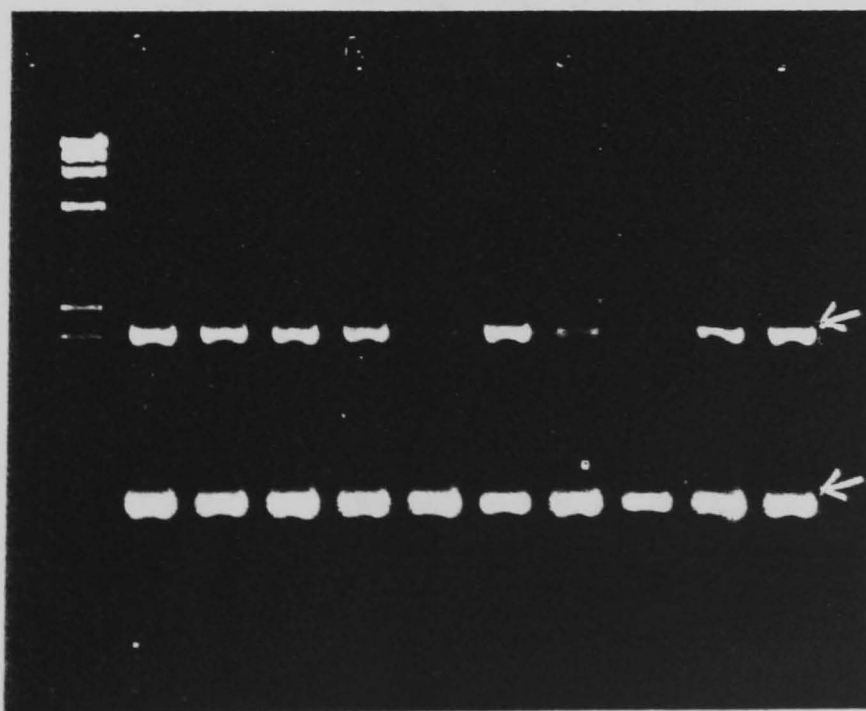


Fig 4.1.1 RAPD profiles of 10 isolates (1-10) of *F. culmorum* produced by Operon primers OPU08, OPU11 and OPU17. λ = Lambda *Hind*III size marker (Kb).

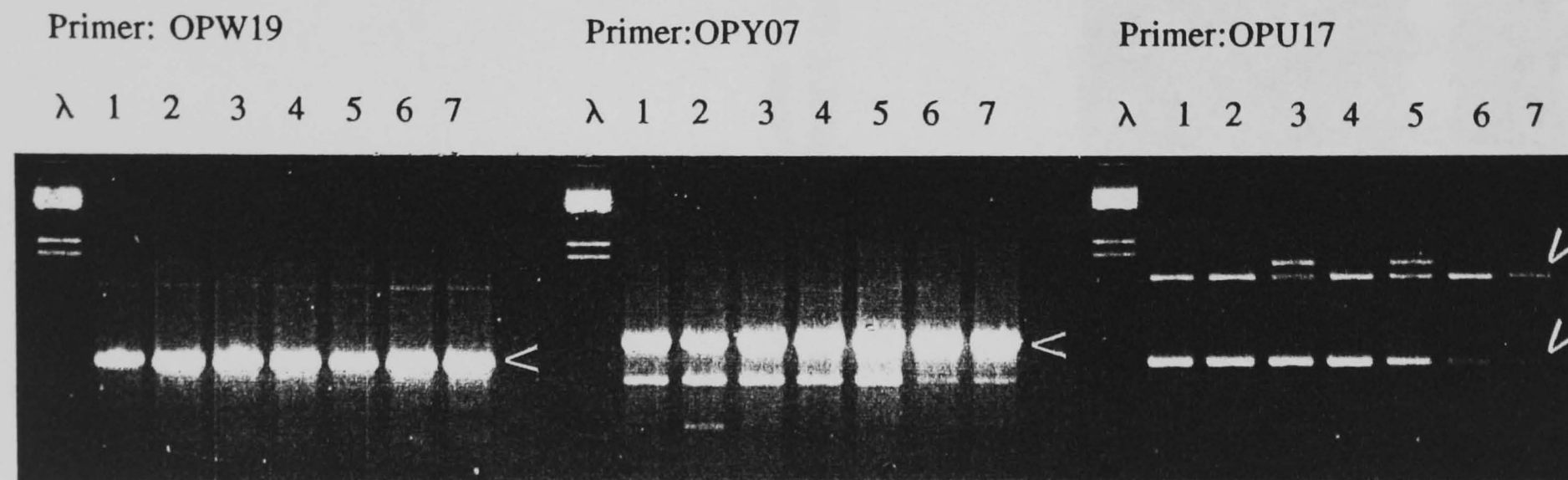


Fig 4.1.2 RAPD profiles of seven isolates (1-7) of *Fusarium avenaceum* produced by Operon primers OPW19, OPY07 and OPU17. λ = Lambda *Hin*III size marker.

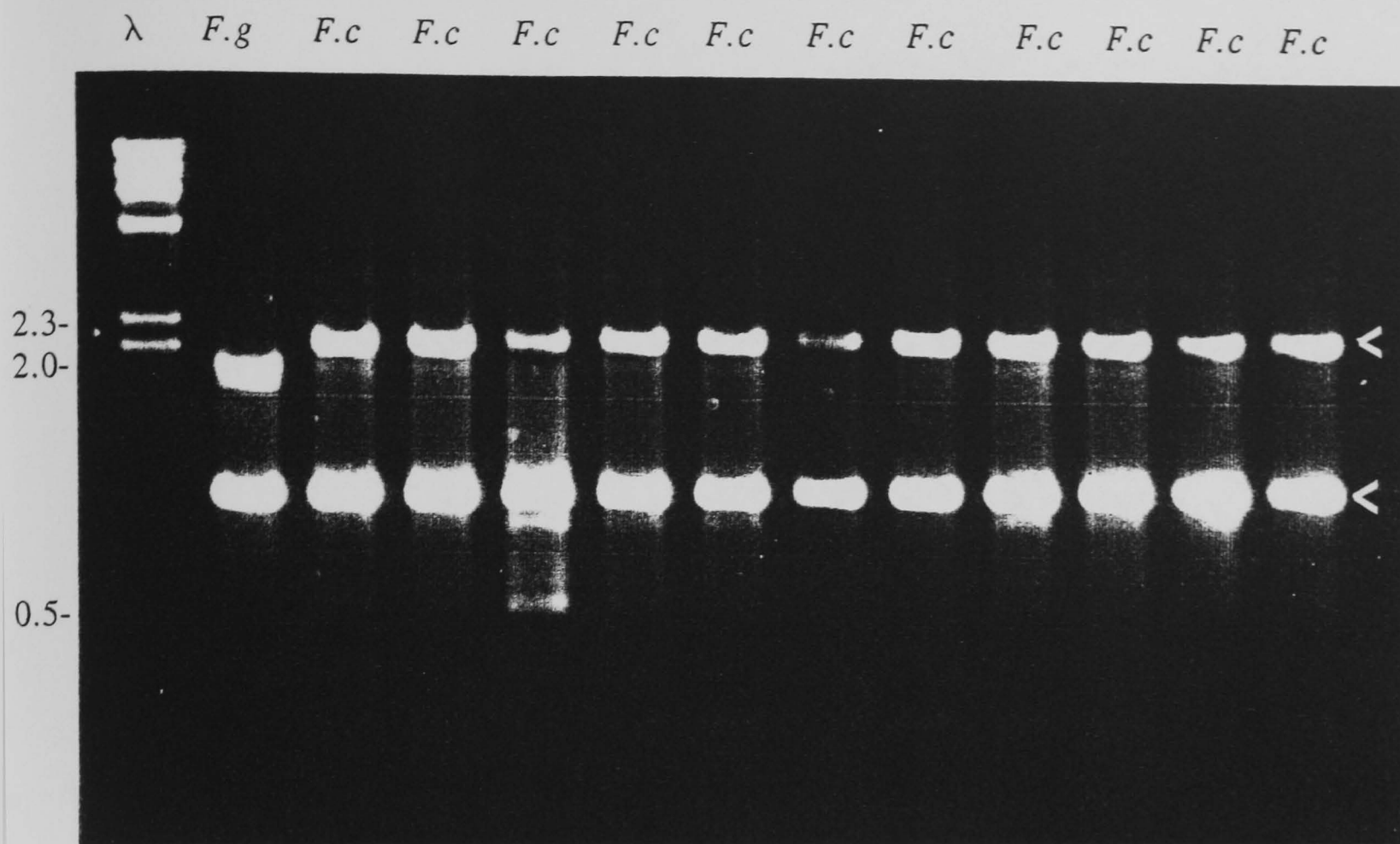


Fig 4.1.3 RAPD profiles of 1 isolate of *F.graminearum* (*F.g*) and 11 isolates of *F.culmorum* (*F.c*) amplified using Operon primer U17. λ = lambda *Hind*III size marker (Kb).

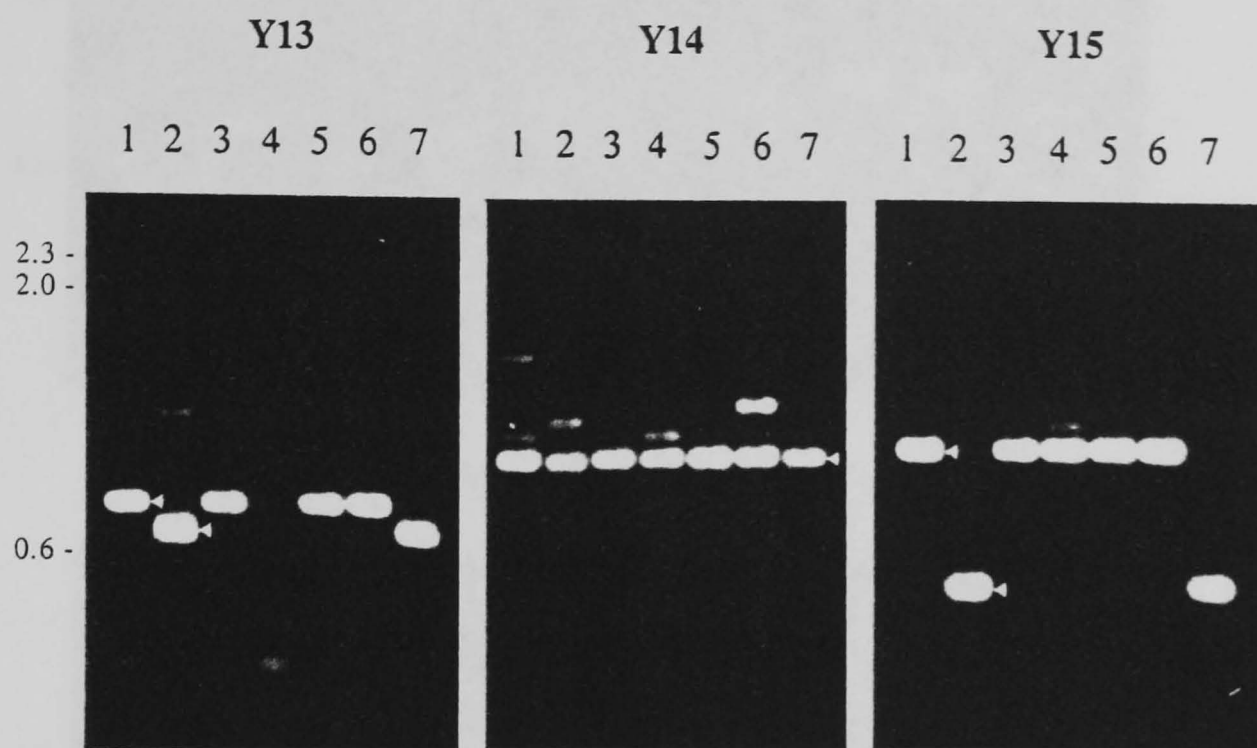


Fig 4.1.4 RAPD profiles of *Microdochium nivale* var.*nivale* (lanes 2 and 7) and *M.nivale* var.*majus* (lanes 1, 3, 4, 5 and 6) produced using Operon primers OPY13, OPY14 and OPY15.

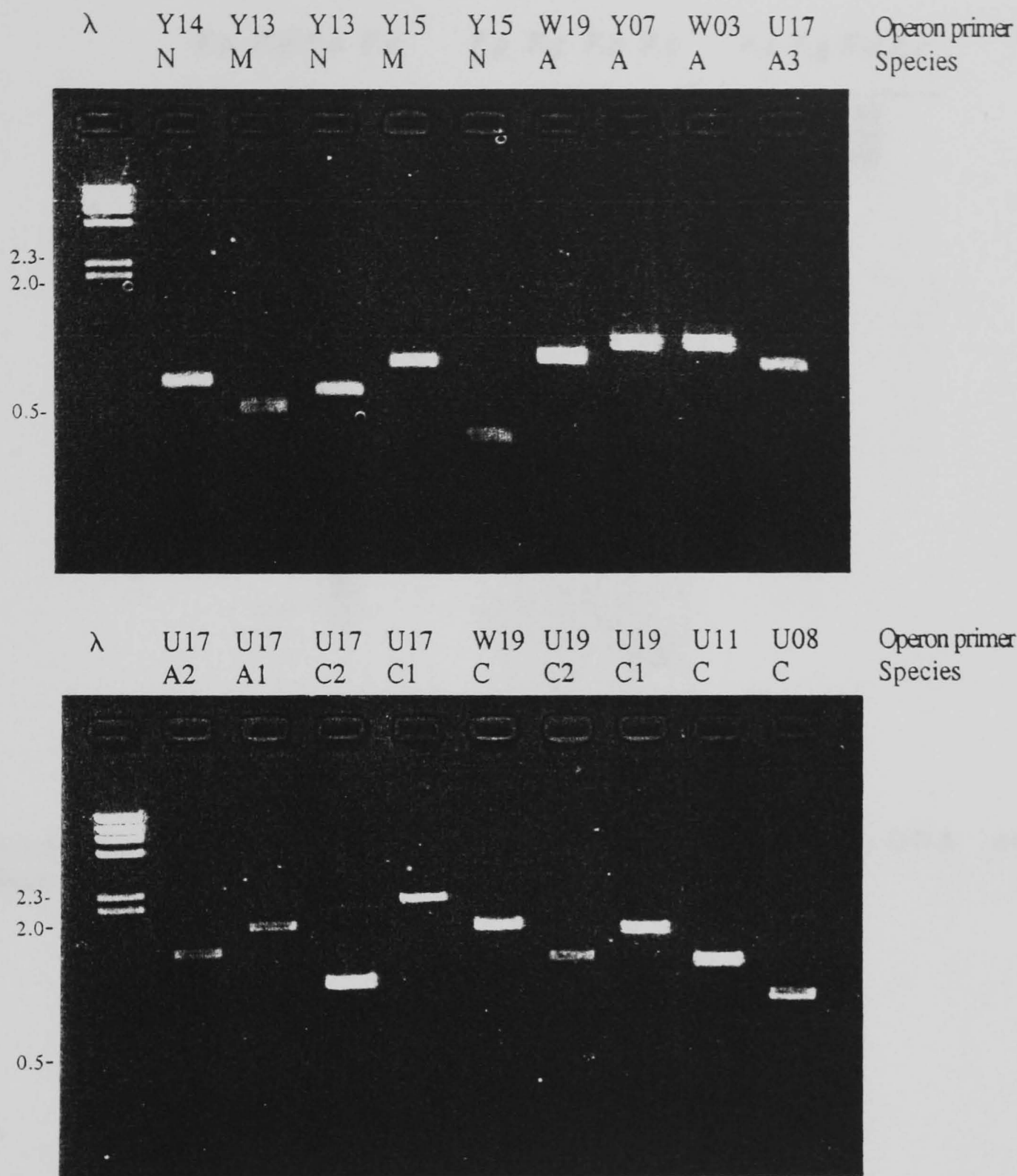


Fig 4.1.5 Purified amplification products of *F.culmorum*, *F.avenaceum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* DNA amplified with selected Operon primers. C = *F.culmorum*, A = *F.avenaceum*, N = *M.nivale* var.*nivale*, M = *M.nivale* var.*majus*. Where more than one band has been selected for each primer, the products are numbered accordingly. λ = lambda HindIII size marker (Kb).

Primer: U17A2
Origin: *F.avenaceum*

Y07A
F.avenaceum

WO3A
F.avenaceum

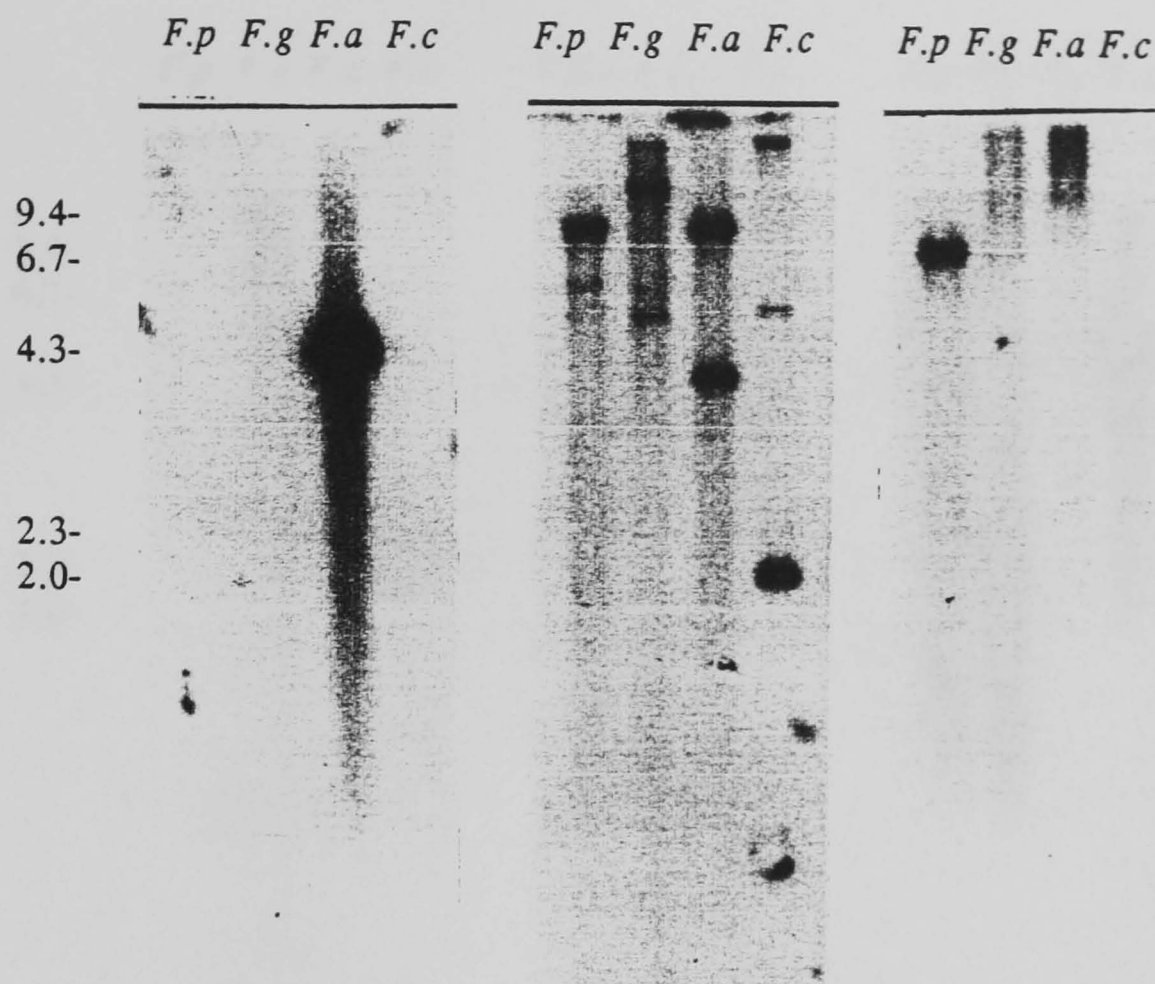


Fig 4.1.6 Hybridisation of RAPD fragments from *F.avenaceum* to total genomic DNA of *F.culmorum*, *F.avenaceum*, *F.graminearum* and *F.poa* restricted with *EcoRI*.

Primer: U08C
Origin: *F.culmorum*

U171C
F.culmorum

U172C
F.culmorum

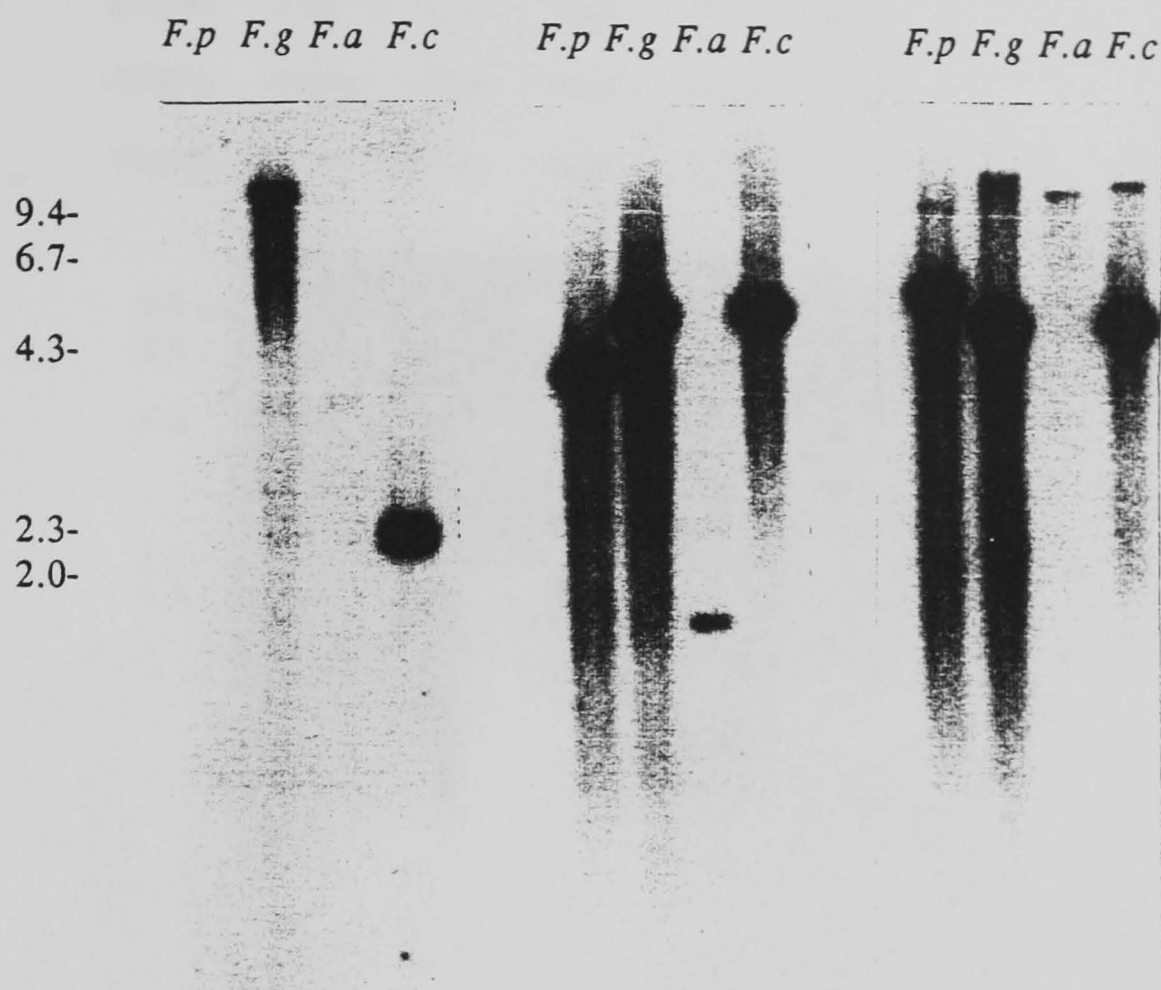


Fig 4.1.7 Hybridisation of RAPD fragments from *F.culmorum* to total genomic DNA of *F.culmorum*, *F.avenaceum*, *F.graminearum* and *F.poa* restricted with *Eco*RI.

primer:	Y13	Y13	Y14	Y15	Y15
origin:	<i>majus</i>	<i>nivale</i>	<i>majus</i>	<i>majus</i>	<i>nivale</i>
variety:	<i>m</i> <i>n</i>	<i>m</i> <i>n</i>	<i>m</i> <i>n</i>	<i>m</i> <i>n</i>	<i>m</i> <i>n</i>

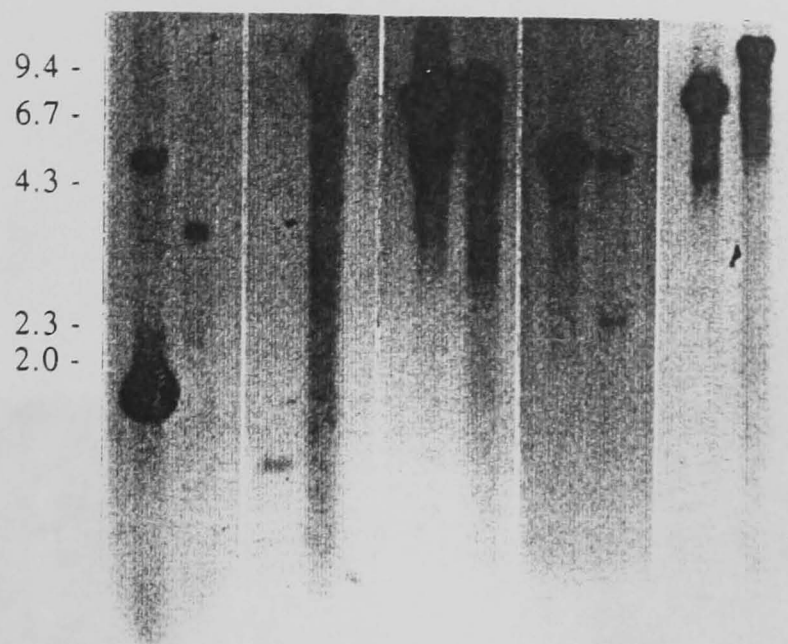
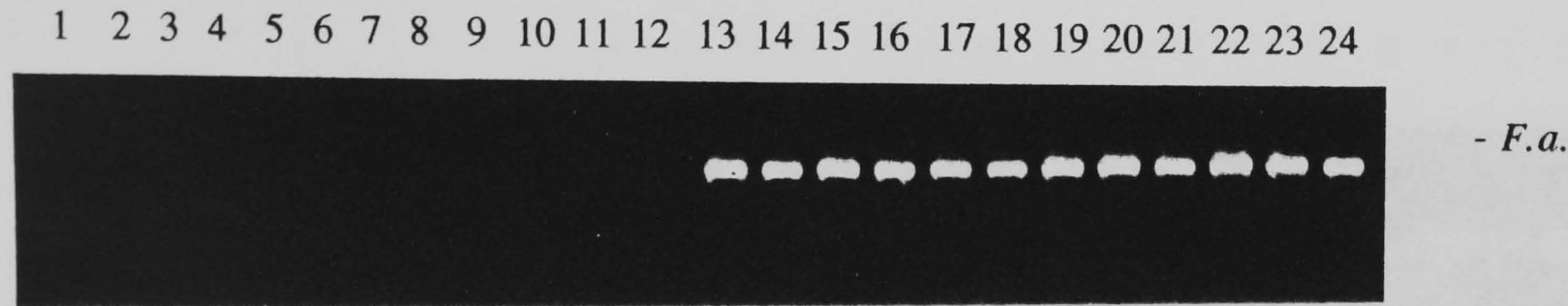


Fig 4.1.8 Hybridisation of RAPD fragments from *Microdochium nivale* var.*nivale* (n) and *M.nivale* var.*majus* (m) produced using Operon primers OPY13, OPY14 and OPY15 to total genomic DNA of both varieties restricted with *Eco* RI.

a)



b)

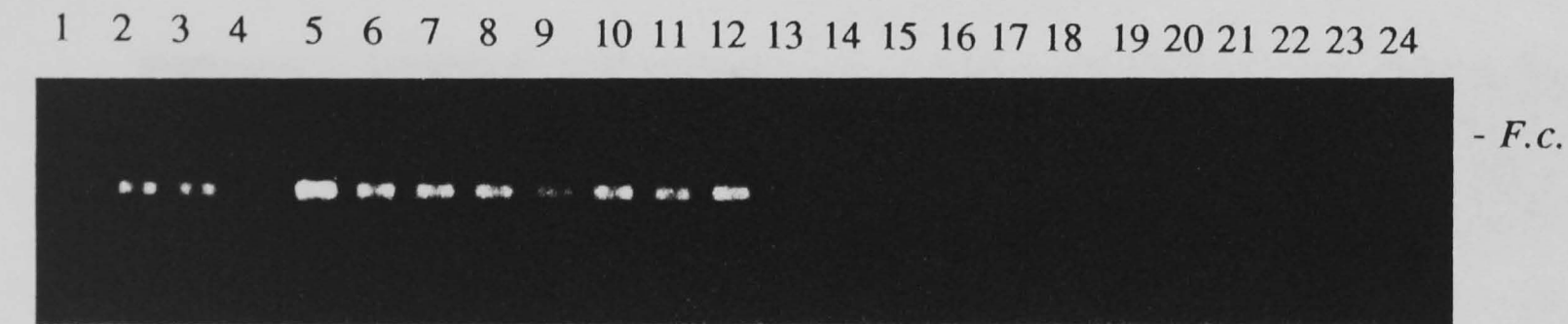


Fig 4.2.1 Amplification of 12 isolates of *Fusarium culmorum* and 12 isolates of *Fusarium avenaceum* using primers specific for a) *Fusarium avenaceum* (*F.a.*) and b) *Fusarium culmorum* (*F.c.*). Isolates 1-12 = *F.c.* isolates, and isolates 13-24 = *F.a.*

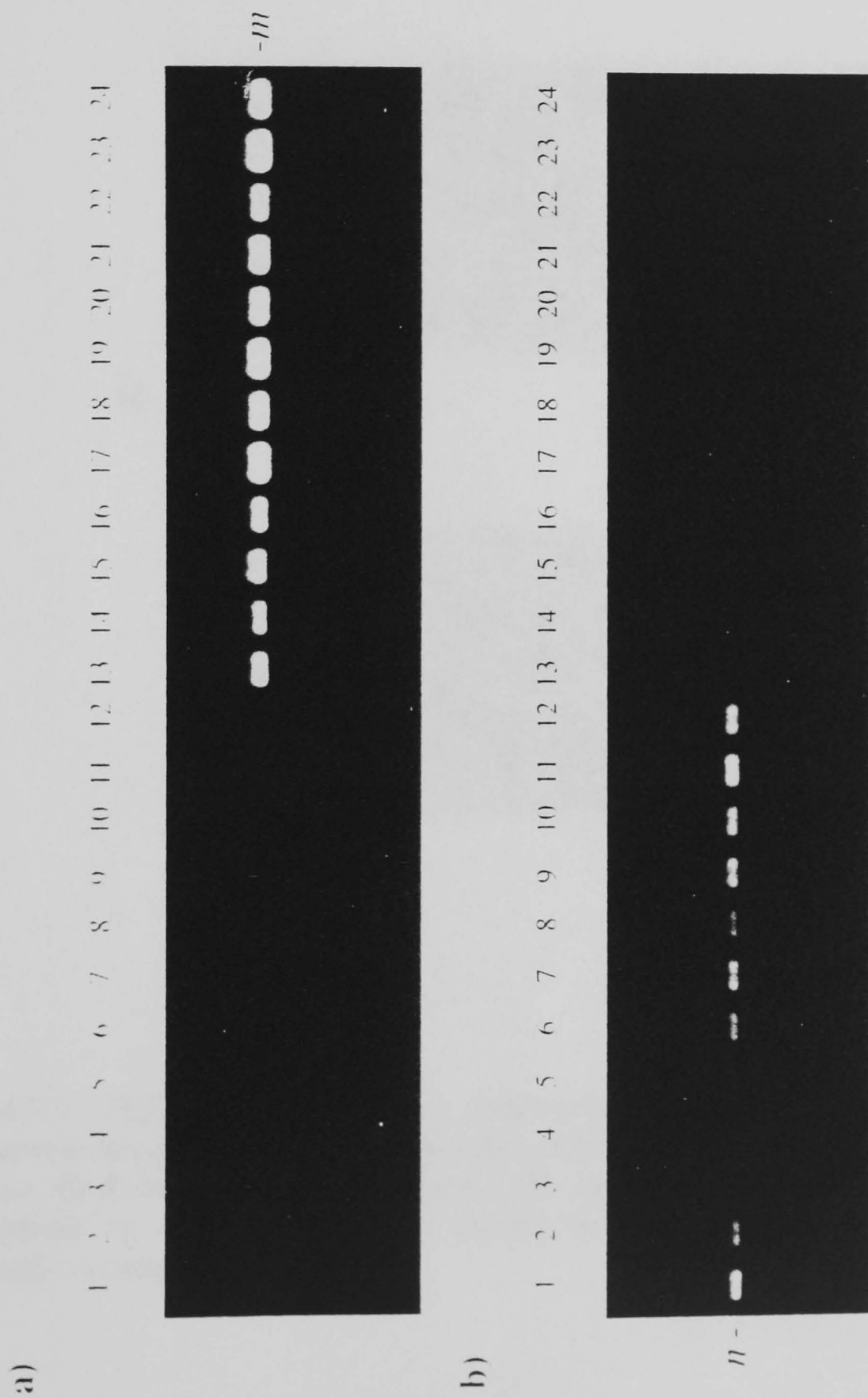


Fig 4.2.2 Amplification of 24 isolates of *Microdochium nivale* using primers specific for a) *M. nivale* var. *majus* (m) and b) *M. nivale* var. *nivale* (n). Isolates 1-12 = *var. nivale* type isolates, and isolates 13-24 = *var. majus*.

a)

1 2 3 4 5 6 7 8 9



b)

1 2 3 4 5 6 7 8 9



Fig 4.2.3. PCR amplification of 1) *Rhizoctonia cerealis*, 2) *Pseudocercospora herpotrichoides* (W-type), 3) *P. herpotrichoides* (R-type), 4) *Fusarium graminearum*, 5) *F. poae*, 6) *F. avenaceum*, 7) *F. culmorum*, 8) *Microdochium nivale* var. *nivale*, and 9) *M. nivale* var. *majus* using primers specific for a) *M. nivale* var. *nivale* (n) and b) *M. nivale* var. *majus* (m).

Species:	<i>M.nivale</i> var. <i>nivale</i>							<i>M.nivale</i> var. <i>majus</i>							<i>F.culmorum</i>								
Dilution:	1	2	3	4	5	6	7	λ	1	2	3	4	5	6	7	λ	1	2	3	4	5	6	7

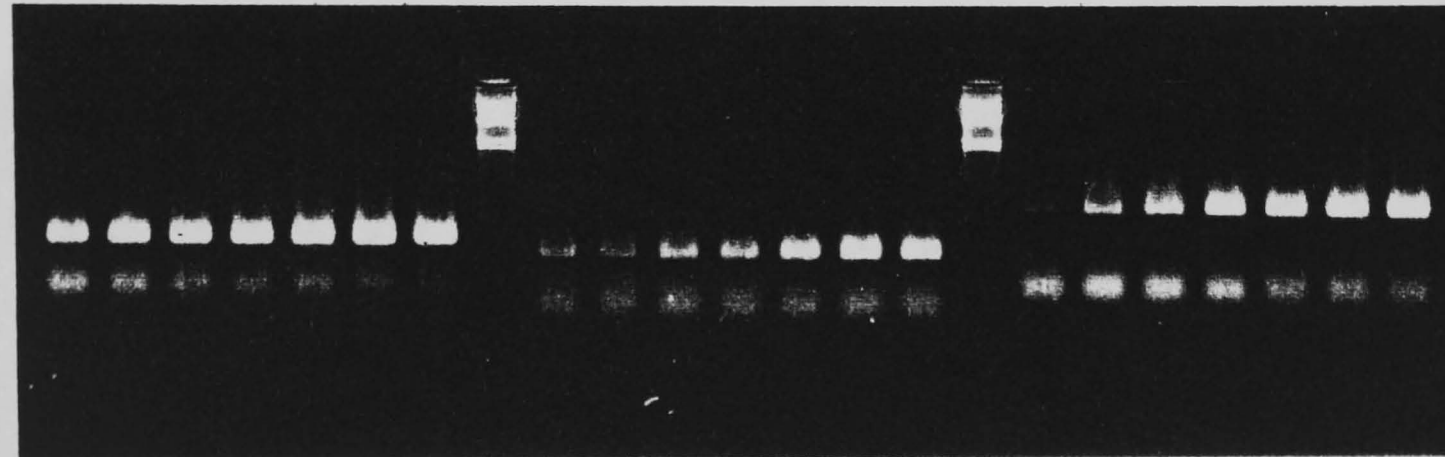
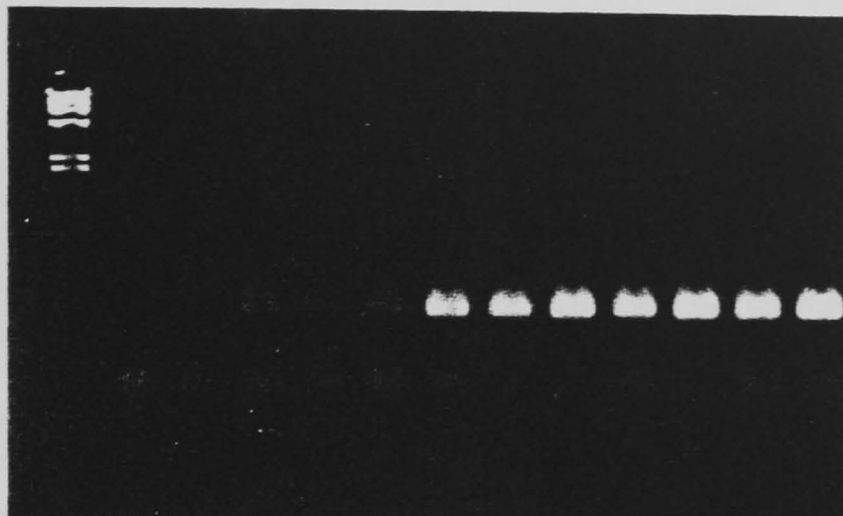


Fig 4.2.4 PCR amplification of diluted pure fungal DNA of *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* (1 = 60 fg/ μ l DNA, 2 = 120fg/ μ l, 3 = 0.23pg/ μ l, 4 = 0.5pg/ μ l, 5 = 1pg/ μ l, 6 = 2pg/ μ l, 7 = 4pg/ μ l) with the relevant species specific primers. λ = lambda *Hind*III size marker (Kb).

Species: *M.nivale* var.*nivale*
Dilution: λ 1 2 3 4 5 6 7 8 9 10 11 12



Species: *M.nivale* var.*majus*
Dilution: λ 1 2 3 4 5 6 7 8 9 10 11 12



Species: *F.culmorum*
Dilution: λ 1 2 3 4 5 6 7 8 9 10 11 12

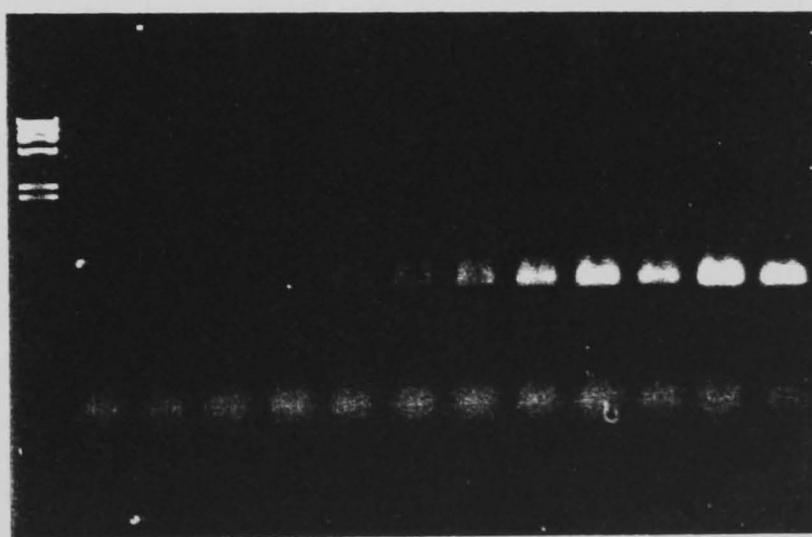


Fig. 4.2.5 PCR amplification of diluted pure fungal DNA of *M.nivale* var.*nivale*, *M.nivale* var.*majus* and *F.culmorum* (1 = 60fg/ μ l DNA, 2 = 120fg/ μ l, 3 = 0.23pg/ μ l, 4 = 0.5pg/ μ l, 5 = 1pg/ μ l, 6 = 2pg/ μ l, 7 = 4pg/ μ l, 8 = 8pg/ μ l, 9 = 16pg/ μ l, 10 = 32pg/ μ l, 11 = 64pg/ μ l, 12 = 120pg/ μ l) in the presence of a background of 100:1 plant:fungal DNA.

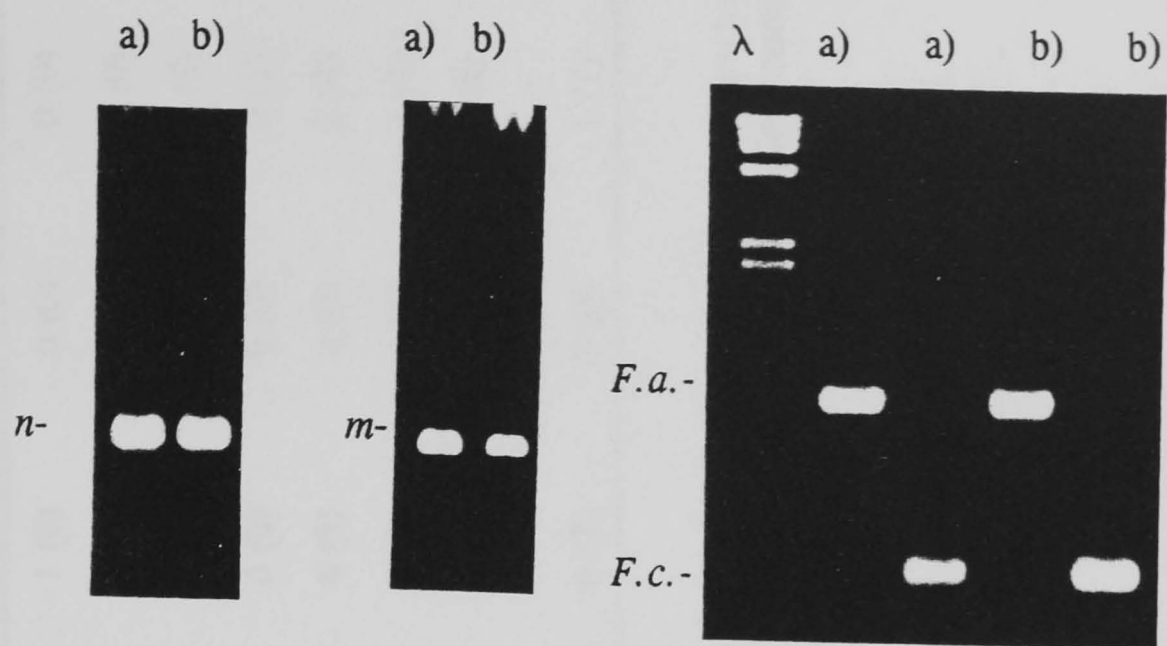


Fig 4.3.1 PCR amplification using primers specific for *M.nivale* var.*nivale* (*n*), *M.nivale* var.*majus* (*m*), *F.culmorum* (*F.c.*), and *F.avenaceum* (*F.a.*) to detect a) pure fungal DNA of each of these species and b) infection of artificially inoculated wheat seedlings. λ = Lambda *Hind*III size marker (kb).

Treatment (species inoculated)	Number of samples infected with each combination of species (n = 8)							
	C	M	N	C+M	C+N	M+N	C+M+N	uninfected
1+(9) <i>F.culmorum</i>	5 (6)	0 (0)	0 (0)	1 (2)	1 (0)	0 (0)	0 (0)	1 (0)
2+(10) <i>M.nivale</i> var. <i>majus</i>	0 (0)	0 (0)	0 (0)	6 (7)	0 (0)	1 (0)	1 (0)	0 (1)
3+(11) <i>M.nivale</i> var. <i>nivale</i>	0 (0)	0 (0)	0 (0)	0 (0)	3 (1)	0 (1)	4 (6)	1 (0)
4+(12) <i>F.culmorum</i> & var. <i>majus</i>	0 (1)	0 (0)	0 (0)	8 (6)	0 (0)	0 (0)	0 (1)	0 (0)
5+(13) <i>F.culmorum</i> & var. <i>nivale</i>	0 (1)	0 (0)	0 (0)	0 (0)	6 (3)	0 (0)	2 (4)	0 (0)
6+(14) var. <i>majus</i> & var. <i>nivale</i>	0 (0)	0 (0)	0 (1)	0 (0)	0 (0)	4 (3)	4 (4)	0 (0)
7+(15) <i>F.culmorum</i> & var. <i>majus</i> & var. <i>nivale</i>	0 (2)	0 (0)	0 (0)	2 (0)	0 (0)	0 (0)	6 (6)	0 (0)
8+(16) uninoculated control	0 (3)	0 (1)	0 (0)	6 (1)	0 (2)	1 (0)	1 (1)	0 (0)

Table 4.3.1 Number of samples infected with *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* and all combinations of these species in each treatment (8 samples per treatment each consisting of 5 plants were taken). Treatments 1-8 were not treated with fungicide, and their corresponding treatments 9-16 received an application of the fungicide flusilazole. C= *F.culmorum*, M= *M.nivale* var.*majus*, N= *M.nivale* var.*nivale*.

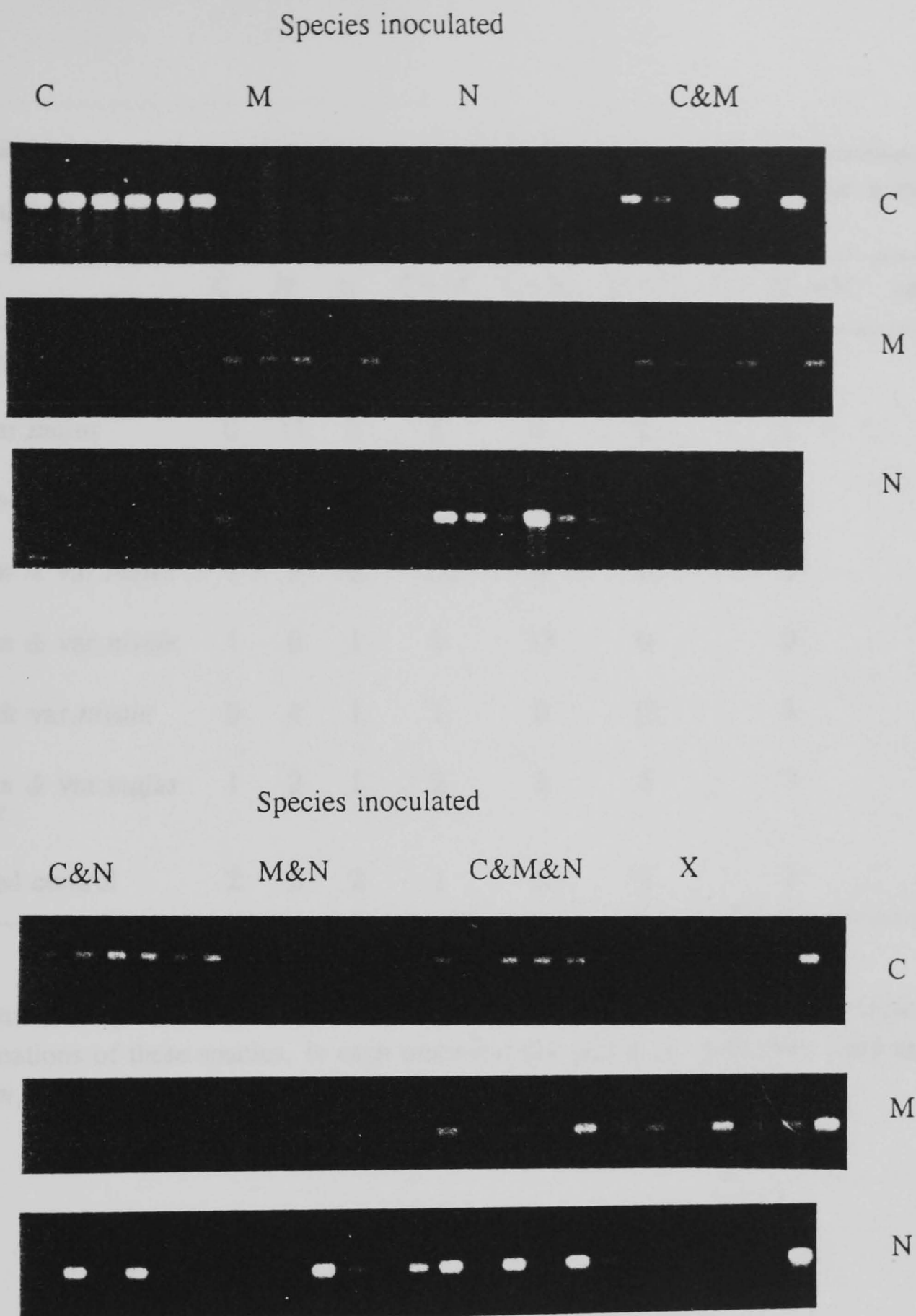


Fig 4.3.2 PCR amplification of DNA extracted from single plants using primers specific for *F.culmorum* (C), *M.nivale* var.*majus* (M) and *M.nivale* var. *nivale* (N). Six individual plants were amplified per treatment, and treatments consisted of inoculations of all combinations of the three pathogens. X represents the six uninoculated control plants.

Treatment (species inoculated)	Number of plants infected with each combination of species. (n = 24)								
	C	M	N	C+M	C+N	M+N	C+ M +N	uninfected	
1 <i>F.culmorum</i>	7	1	1	1	9	0	0	5	
2 <i>M.nivale</i> var. <i>majus</i>	0	11	0	5	0	2	1	5	
3 <i>M.nivale</i> var. <i>nivale</i>	0	0	16	0	3	0	0	5	
4 <i>F.culmorum</i> & var. <i>majus</i>	1	3	0	13	0	0	2	5	
5 <i>F.culmorum</i> & var. <i>nivale</i>	1	0	1	0	13	0	0	9	
6 var. <i>majus</i> & var. <i>nivale</i>	0	4	1	1	0	10	3	5	
7 <i>F.culmorum</i> & var. <i>majus</i> & var. <i>nivale</i>	1	2	1	2	5	5	7	1	
8 uninoculated control	2	0	2	1	5	1	2	7	

Table 4.3.2 Number of plants infected with *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* and all combinations of these species, in each treatment (24 plants per treatment were sampled). C = *F.culmorum*, M = *M.nivale* var.*majus*, N = *M.nivale* var.*nivale*.

Species and category	Number of plants in each category for each treatment (n = 24)							
	Treatment number							
	1	2	3	4	5	6	7	8
	C	M	N	C&M	C&N	M&N	C&M&N	CONTROL
<i>F.culmorum</i>								
0	7	18	21	8	10	20	9	14
1	0	2	3	0	0	1	0	4
2	2	3	0	1	2	2	2	6
3	15	1	0	15	12	1	13	0
<i>M.nivale</i> var. <i>nivale</i>								
0	14	20	5	22	10	9	6	14
1	5	3	2	2	2	1	5	7
2	1	0	3	0	4	5	3	3
3	4	0	14	0	8	9	10	0
<i>M.nivale</i> var. <i>majus</i>								
0	21	5	24	6	24	5	8	20
1	2	9	0	9	0	7	11	2
2	0	10	0	9	0	12	3	2
3	0	0	0	0	0	0	2	0

Table 4.3.3 Number of plants infected with *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* in each of the brightness catagories 0, 1, 2 and 3 where 0 = no band, 1 = faint band, 2 = moderately bright band and 3 = bright band for treatments 1-8. A total of 24 individual plants were sampled per treatment.

DISCUSSION

Random amplified polymorphic DNA (RAPD) assays have been used widely to analyse variation in organisms by the detection of polymorphism as described by Williams *et al.* (1990). Welsh & McClelland (1990) used a RAPD assay to show that strains of *Streptococcus* could be distinguished by a comparison of polymorphisms in genomic fingerprints, and Guthrie *et al.* (1992) and Van der Vlugt-Bergmans *et al.* (1992) analysed variability in *Colletotrichum graminicola* and *Botrytis cinerea* respectively, and were able to differentiate isolates using this method.

In this study however, RAPDs were used to identify non-polymorphic markers to an individual species. RAPD analysis provided an efficient method of selecting random species specific primers, as polymorphisms between species could be quickly identified by screening of primer kits. Several of the primers selected by RAPD analysis which showed polymorphisms between the *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* species showed no polymorphism when tested against a range of isolates of the relevant pathogen, and were therefore selected for further development.

DNA probes for the detection and identification of various pathogens have been described by other workers. For example, Henson (1989) generated a probe for the identification of *Gauemannomyces graminis*, and Ward & Gray (1992) later developed probes able to differentiate three varieties of this pathogen. Nicholson *et al.* (1994) identified a pathotype specific DNA probe for the R-type of *P.herpotrichoides*, and Yao *et al.* (1991) were able to detect and identify *Peronosclerospora sacchari* using DNA probes.

In the current work, DNA fragments exhibiting hybridisation to *F.culmorum*/*F.graminearum*, *F.avenaceum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* have been isolated. The fragments did not hybridise to DNA extracted from isolates of other stem base pathogens. The cross hybridisation between isolates of *F.culmorum* and

F.graminearum illustrates the close relationship between the two species, both of which belong to the discolor section of the *Fusaria* (Booth 1971). Both species can occur in the foot rot disease complex, although *F.culmorum* is much more commonly isolated from stem bases in the U.K. than *F.graminearum* (Polley *et al.*, 1991). Further work may be required to produce probes specific for each of these species, but in the current work, the ability to discriminate between the two species was not considered to be critical due to the low incidence of *F.graminearum* reported in the stem base.

Sequencing of the selected DNA sequences and the use of the 'primer' program to identify primers, which were then constructed was successful in producing longer oligonucleotides of known sequence which were able to detect the pathogens *F.avenaceum*, *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* with no cross specificity between species. Use of the species specific *M.nivale* var.*nivale* and var.*majus* primers produced the first evidence that the *M.nivale* var.*nivale* RAPD group 2 (as discussed in chapter 3.0) is a single grouping, as PCR primers to *M.nivale* var.*nivale* (derived from isolate Mn1) amplified all tested isolates of the highly variable RAPD defined group 2 of *M.nivale*. This was subsequently supported by the work of Parry *et al.* (1995a) who amplified over 200 isolates of *M.nivale*, using the species specific primers and found that all could be classified into one of the two groups. These results were in contrast with those reported for antibodies, as previously discussed for *M.nivale* (Hoxter *et al.*, 1991), and *P.herpotrichoides* Dewey (1988), Unger & Wolf (1988) where problems with cross specificity and an inability to discriminate pathotypes were reported.

The species specific PCR primers, when tested against pure fungal DNA, produced bright amplification products, and under the PCR conditions used, the degree of amplification was apparently dependent upon the amount of template DNA included in the PCR reaction. The species specific primers were also shown to detect *F.culmorum*,

M.nivale var.*nivale* and *M.nivale* var.*majus* in plant material and there was no loss of specificity induced by the presence of plant DNA in a 100:1 ratio with fungal DNA. The relationship between the amount of fungal DNA and degree of amplification was also apparent in the presence of plant DNA.

DNA quantification using conventional PCR is possible under certain circumstances. However, it is necessary to identify the linear range of the PCR reaction, where the amount of amplified target DNA is proportional to the initial amount of target molecules in order to quantify the reaction accurately (Ferre, 1992). The relationship between the amount of fungal DNA and the brightness of amplification although reproducible with all 3 species tested cannot be used as a measure of the relative amount of DNA present, as amplification of the fragments may not be constant in separate PCR runs, and band intensity cannot therefore be compared across tests. For example, substances such as clay and humic material which may frequently be associated with stem base samples, have been shown to inhibit PCR (Bej & Mahbubani, 1992).

Further experiments using the species specific primers were conducted to examine the infection of seedlings, the competitive ability of isolates of *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus*, and the effect of a fungicide application on the incidence of each species.

In the first test, the application of flusilazole to inoculated seedlings did not reduce the final number of infected plants in any of the treatments. *F.culmorum* was detected in seedlings where it had been inoculated alone, but there was little or no detection of *M.nivale* var.*nivale*, and *M.nivale* var.*majus* where they had been inoculated alone. However, both var.*nivale* and var.*majus* were detected in plants that had also been inoculated with *F.culmorum*. It is possible that 10°C, at which this test was conducted, was sub-optimal for the infection of seedlings where they were inoculated with only

M.nivale var.*nivale* or *M.nivale* var.*majus*. The results from this test suggest that infection and colonisation by *M.nivale* var.*nivale* and var.*majus* may be facilitated by the presence of *F.culmorum*. Background infection/cross contamination was noted, and the control treatment (treatment 8) in particular had a high incidence of *M.nivale* var.*majus* and *F.culmorum* which may have been due to the close proximity of pots within blocks facilitating the splash dispersal of conidia during watering. Contamination by each of the three pathogens was relatively high, many samples were infected with *F.culmorum* and many with *M.nivale* var.*majus*. As each sample in this experiment consisted of a bulk of five plants, it was difficult to assess whether the high levels of pathogen detection were due to widespread infection, or due to single highly infected individuals. It was noted that those plants that had received the artificial inoculum produced bands of a higher intensity than the contaminated, or previously infected plant material.

The second multiple inoculation experiment revealed that the primers were efficient at detecting *F.culmorum*, *M.nivale* var.*nivale* and var.*majus*, and as single plants were sampled in this test, it was possible to determine exactly how many seedlings were infected. However, it appeared that each pathogen was able to colonise wheat seedlings in conjunction with the others. In contrast to the previous experiment (conducted at 10°C), isolates of *M.nivale* var.*nivale* and *M.nivale* var.*majus* were able to infect wheat seedlings when inoculated alone at 15°C. Further work is required to determine the rôle of temperature and presence of *F.culmorum* in the infection and colonisation of seedlings by these two pathogens.

Amplification was also seen in uninoculated plants and can be attributed to seed or soil borne inoculum, as seedlings were grown in isolated pots. Background levels of var.*majus* were considerably lower than those of the other two species, and the reasons for this are unknown. *M.nivale* var.*majus* may be generally less common in the

environment than var.*nivale*, but previous work (see Chapter 3.0) has indicated that this may not be the case, and Parry *et al.* (1995b) found a much higher incidence of var.*majus* than var.*nivale* when they made a national survey of wheat stem base disease. The inoculated plants again produced bands with a higher intensity than the uninoculated, but infected plants. As the amount of fungal DNA present in a sample has been shown to affect intensity of bands, it may indicate that a higher fungal biomass was present in the artificially inoculated material, suggesting that infection and colonisation of the plants had been achieved by the inoculum, as opposed to a surface infection, or contamination. It has been shown (Colhoun *et al.*, 1968), that the level of inoculum of *Fusarium* species present is important in the infection process, and that little seedling disease occurs until a minimum inoculum level is obtained. This may partially explain the low intensity bands of the 'naturally' infected samples, and as the inoculum plug used probably provided a very high inoculum source and nutrient base this may also therefore explain why the infection and colonisation of the plant was successful.

In this study, RAPDs were used to provide markers from which species specific DNA probes, and oligonucleotides for use in PCR were developed. However, the amount of fungal DNA could not be measured by a comparison of band intensity, as many factors can affect the amplification of DNA in the PCR reaction. Therefore, it was considered that further work should be undertaken to develop a system for the quantification of the amount of DNA present in each sample by a relative comparison of the band intensity compared to an internal standard. This work is described in Chapter 5.0.

Chapter 5.0 The quantification of DNA from *Fusarium culmorum*, *Microdochium nivale* var. *nivale* and *Mnivale* var. *majus* using a competitive Polymerase Chain Reaction assay.

Many attempts to quantify fungal biomass have been made. For example, as early as 1948 the quantitative estimation of soil micro-organisms using an agar film method was attempted (Jones & Mollison, 1948). This method was not however suitable for the determination of fungal mycelia in tissue and chemical methods of quantification based on the colorimetric assay of chitin were adopted. Ride & Drysdale (1971) used a method based upon enzyme hydrolysis to estimate the mass of *Fusarium oxysporum* f. *lycopersici* in infected tomato plants, and were able to measure a minimum of 5 µg fungus/g tissue. The chitin assay technique for estimation of fungal biomass was questioned by Sharma *et al.* (1977), who showed that chitin content of mycelium varied with cultural conditions, and that the technique was therefore of limited use. Further work concentrated on ergosterol measurements as a means of fungal quantification. Ergosterol tends to be restricted to fungi (Weete & Weber, 1980), and is unstable, providing a good indicator of fungal cell viability. Gretenkort & Ingram (1993) found a good correlation between disease scores in *Brassica napus* caused by *Leptosphaeria maculans* and ergosterol levels. However, these workers found problems with variation between replicates in plant material. Newton (1989) found that sterol analysis was an efficient method of comparing barley resistance to *Erysiphe graminis* f.sp. *hordei* as the sterol content was highly correlated with the amount of mildew estimated by infection frequency and colony area, but noted that the identity of the molecule producing sterol is not known and therefore cannot be unequivocally regarded as a component of the mildew mycelium. Bermingham *et al.* (1995) examined the relationship between ergosterol content and biomass of nine species of aquatic hyphomycetes and found that

ergosterol was an unreliable method of measuring total biomass even in species for which direct relationships between ergosterol and biomass occur. The study showed that there was no simple relationship between ergosterol content and biomass. This lack of relationship, a high level of interspecific variation and the dependence of ergosterol content on culture conditions brings into question the efficacy of this technique.

The use of monoclonal antibody immunoassays for the detection of *Pseudocercospora herpotrichoides* has been demonstrated (Priestley & Dewey, 1993). These authors suggested that such an assay could be used for quantitative measurements of the pathogen within infected host tissue, but did not provide evidence to substantiate this claim. Unger & Wolf (1988) found that detection of the eyespot pathogen *P. herpotrichoides* in wheat using enzyme linked immunosorbent assay (ELISA) was correlated to visual disease indices, and concluded that this was a reliable method of quantitative detection. However, due to the inability of their system to differentiate between W and R-type eyespot, and the difficulty in differentiation of stem base disease symptoms, these results may be questionable.

DNA amplification using species specific primers in the Polymerase Chain Reaction has been shown to be an effective method for the sensitive detection of many organisms (see chapter 4.0). However, most PCR based assays are qualitative rather than quantitative, and are therefore restricted to applications where detecting the absence or presence of a specific DNA molecule is sufficient. Despite the ability of the assay to detect very small amounts of the target DNA, accurate quantification is difficult due to the nature of the PCR reaction. As DNA is replicated exponentially during the PCR reaction, it is necessary to determine the initial concentration of DNA present by an extrapolation from the final concentration of DNA produced in the reaction. However, quantitative PCR has been developed for use with a wide variety of systems.

Warren *et al.* (1991) used High Performance Liquid Chromatography (HPLC) to

separate PCR products, and by comparing the optical density of these products with a calibration were able to quantify a minimum of 1ng of DNA using this method. Landgraf *et al.* (1991), described a method of labelling PCR primers with biotin and fluorescent dye, in order to quantify the PCR products by a comparison with the template concentration. Similarly, Murphy *et al.* (1990) attempted to quantify gene expression by the same method, in this case using radiolabelled primers. The radiolabelled PCR product was then quantified using scintillation counting of bands excised from a gel, and by densitometric scanning of bands after autoradiography. Hayashi *et al.* (1989) used ^{32}P labelled primers to detect PCR products, and compared the relative intensities of the bands produced after 25 PCR cycles with the template concentration. These procedures required exact standardisation, and were only feasible during the logarithmic phase of the reaction when the concentration of *Taq* polymerase was not limiting. Since the determination of the exponential phase was found to be labour intensive, and extended only a few cycles above the level of detection of the signal (Murphy *et al* 1990), this method was not found to be ideal.

A quantitative solid-phase approach to the detection of immobilized, amplified nucleic acids (DIANA) was taken by Lundeberg *et al.* (1991), who produced competitor DNA fragments, identical to the target DNA, but carrying an extra 21 base pair sequence detectable by the addition of a protein to the reaction. By a colorimetric comparison of the amount of competitor to target DNA produced during the PCR reaction these workers were able to quantify the amount of target DNA initially present. Similar methods involving the coamplification of the molecule of interest with an internal standard having identical forward and reverse primers have been described. The target DNA and the control fragment compete for the same primers, and therefore, the ratio of amplification product of target and control fragment relates to the ratio of the input target DNA and a known quantity of control DNA in the reaction. The internal standard

should be a DNA molecule that can be amplified by the same primer pair as the target, but yield a product that is easily distinguished from the counterpart. During the exponential phase of PCR, the ratio of the target DNA and the competitor DNA should remain constant, eliminating the error caused by differences in efficiency of amplification. Methods for the synthesis of internal standards for competitive PCR are documented by Celi *et al.* (1993) Jin *et al.* (1994) and Förster (1994). Überla *et al.* (1991) used fragments from distantly related species to construct competitive sequences having the same primer ends as the target DNA. This reaction was semi-quantitative, due to possible differences in reannealing behaviour or amplification rates of the two fragments. According to Simon *et al.* (1992) the use of similar DNA sequences minimises bias in amplification, and interference with unwanted sequences is minimised. Porcher *et al.* (1993) also noted that the use of internal standards closely related to the target sequence ensures that the efficiency of amplification of the internal standard remains identical to that of the sequence of interest after the exponential phase of PCR. Internal control DNA templates for standardisation are also useful as indicators of inhibition or reaction failure, and provide a baseline from which comparisons can be made. The use of internal standards for quantification has been demonstrated. Wang *et al.* (1989) used a synthetic RNA as an internal standard to quantify mRNA, and Simon *et al.* (1992) produced an internal standard to quantify endomycorrhizal fungi colonising leek roots. Hu *et al.* (1993) showed that PCR could be used to quantify the fungal biomass of *Verticillium in vivo*. These workers were able to quantify template DNA in the range 0.0001-100ng using radiolabelled PCR products, detected by scintillation counting. Moukhamedov *et al* (1994) were also able to quantify *Verticillium tricorpus* in diseased potato plants using an internal standard.

The development of a quantitative system for the measurement of fungal biomass of the stem base disease pathogens would be beneficial for epidemiological studies of the

pathogens, studies of fungicide efficacy and the examination of competition between species. In this work, the PCR assay developed for the detection of stem base disease components was adapted for the quantification of *F.culmorum* and *M.nivale* in wheat plant tissue according to the method of Förster (1994), by which the competitive standard of defined size for a chosen PCR product is generated in two consecutive reamplifications. The species specific *F.culmorum* and *M.nivale* primers were used to produce internal control templates. The original templates were partially deleted to produce shorter products which could be differentiated from the fungal amplification product by gel electrophoresis, and could be quantified using densitometric scanning, avoiding the requirement for the use of radioisotopes.

MATERIALS AND METHODS

Development of internal standard sequences using PCR

Amplification reactions were performed in volumes of 50 μ l containing genomic template DNA of either *F.culmorum*, *M.nivale* var.*nivale* or *M.nivale* var.*majus*. The reaction buffer consisted of 100 μ M each of dATP, dCTP, dGTP and dTTP, and 0.8 units of *Taq* polymerase (Boehringer Mannheim Ltd) in 10mM Tris; HCl (pH 8.3), 1.5mM-MgCl₂, 50mM-KCl, 100 μ g ml⁻¹ gelatine, 0.05 % Tween 20 and 0.05 % Nonidet-P40. Reaction mixtures were overlaid with mineral oil prior to PCR.

Amplification was performed in a Perkin Elmer Cetus DNA thermal cycler programmed for 40 cycles of 30 sec at 95°C, 20 sec at 60°C and 45 sec at 72°C using the fastest possible transition times. In the first amplification reactions, the forward and reverse *F.culmorum* and *M.nivale* var.*nivale* and var.*majus* species specific primers were used to amplify target DNA from an isolate of the relevant species. A 10 μ l aliquot of each amplification product was fractionated by electrophoresis through a 1.5 % agarose gel, and detected by staining with ethidium bromide to ensure that amplification had

occurred, and that the band produced was of the expected size in each case.

In the first re-amplification step, 1 μ l of a 1:10³ dilution of the previously amplified PCR product was used as a template, and the 3' primer used in the preceding reaction was replaced by an internal linker primer (see Fig 5.1.1). This internal linker primer corresponded to a 16-17b.p. sequence approximately 50bp upstream of the 3' primer annealing site. The linker primer also had a tail of 10 bases at the 5' end which was identical to the first ten bases at the 3' end of the 3' primer, therefore acting as a linker between the two primers in the second re-amplification step. In all reactions, the same 5' primer was used. Following the first PCR re-amplification, a 10 μ l aliquot of the product was compared to the product of the original amplification by electrophoretic gel separation, to ensure that the product of the first re-amplification was approximately 50 base pairs smaller than the product amplified from the fungal template DNA. In the final (second) re-amplification, 1 μ l of a 1:10³ dilution of the reaction mixture containing the smaller fragment obtained from the first re-amplification was amplified using the original 3' and 5' species specific primers. A 10 μ l aliquot of the resulting PCR product was compared to the products of the previous amplifications. In this case, the PCR product was smaller than the original fungal DNA template, but was assumed to have identical 3' and 5' end sequences to the target DNA. This fragment was then taken as the competitive standard for use in PCR reactions. The amplification steps taken to produce the internal standard are depicted in Fig 5.1.1.

Calibration of the internal standard

A 15 μ l aliquot of the internal standard fragment PCR product for each species was fractionated by gel electrophoresis through a 3% low melting point agarose gel, and visualised by staining with ethidium bromide. The resulting bands were excised from the gel and diluted in 100 μ l H₂O by heating to 70°C for 30 secs. The internal standard

of each species was diluted further with water to dilutions of 0.5×10^3 , 0.25×10^3 , 0.125×10^3 , 0.06×10^3 , 1.0×10^4 , 0.5×10^4 , 0.25×10^4 , 0.125×10^4 and 0.06×10^4 . Each dilution of the internal standard was coamplified with $1\mu\text{l}$ of fungal DNA (5ng) in a PCR reaction as described previously. The resulting PCR products were then electrophoresed through a 1.5% agarose gel, and the concentration of the internal standard producing a signal strength equivalent to 5ng of fungal DNA for each species was noted, and used in further dilutions.

A $1\mu\text{l}$ aliquot of the internal standard chosen for each species was then amplified using PCR in the presence of a range of DNA concentrations of the relevant pathogen. These PCR products were electrophoresed through 2% agarose gels stained with ethidium bromide and photographed under UV illumination with Polaroid 665 positive/negative film. The resulting photographic negatives were scanned using a Chromoscan 3 densitometer (Vickers Co. Malden, USA). Following scanning of negatives taken of the products from each dilution series the relationship between product ratios and the amount of fungal DNA was determined.

Quantification of seedling infection by *F.culmorum*

DNA extracted from wheat seedlings cv. Mercia inoculated with either *F.culmorum*, *M.nivale* var. *nivale*, or *M.nivale* var. *majus*, or a combination of these species which had previously been tested for the presence of each of these pathogens using species specific primers in a PCR assay (for results see Chapter 4. 3) was used as the target DNA in a competitive PCR reaction. A $1\mu\text{l}$ aliquot of DNA from each of the six samples in treatments 1 (*F.culmorum* inoculated), 4 (*F.culmorum* & var.*majus* inoculated), 5 (*F.culmorum* and var.*nivale* inoculated), 7 (*F.culmorum*, var.*majus* and var.*nivale* inoculated) and 8 (uninoculated) was amplified in a PCR reaction as described previously in the presence of $1\mu\text{l}$ of the *F.culmorum* internal standard DNA.

Three replicates of each sample were tested. The resulting PCR products were electrophoresed through 2% agarose gels stained with ethidium bromide and viewed under UV illumination. Polaroid 665 positive/negative photographs were taken of all samples. These negatives were then scanned as before using densitometry, and the amount of fungal DNA present in each sample calculated by a comparison with the calibration curve previously developed with known amounts of fungal DNA.

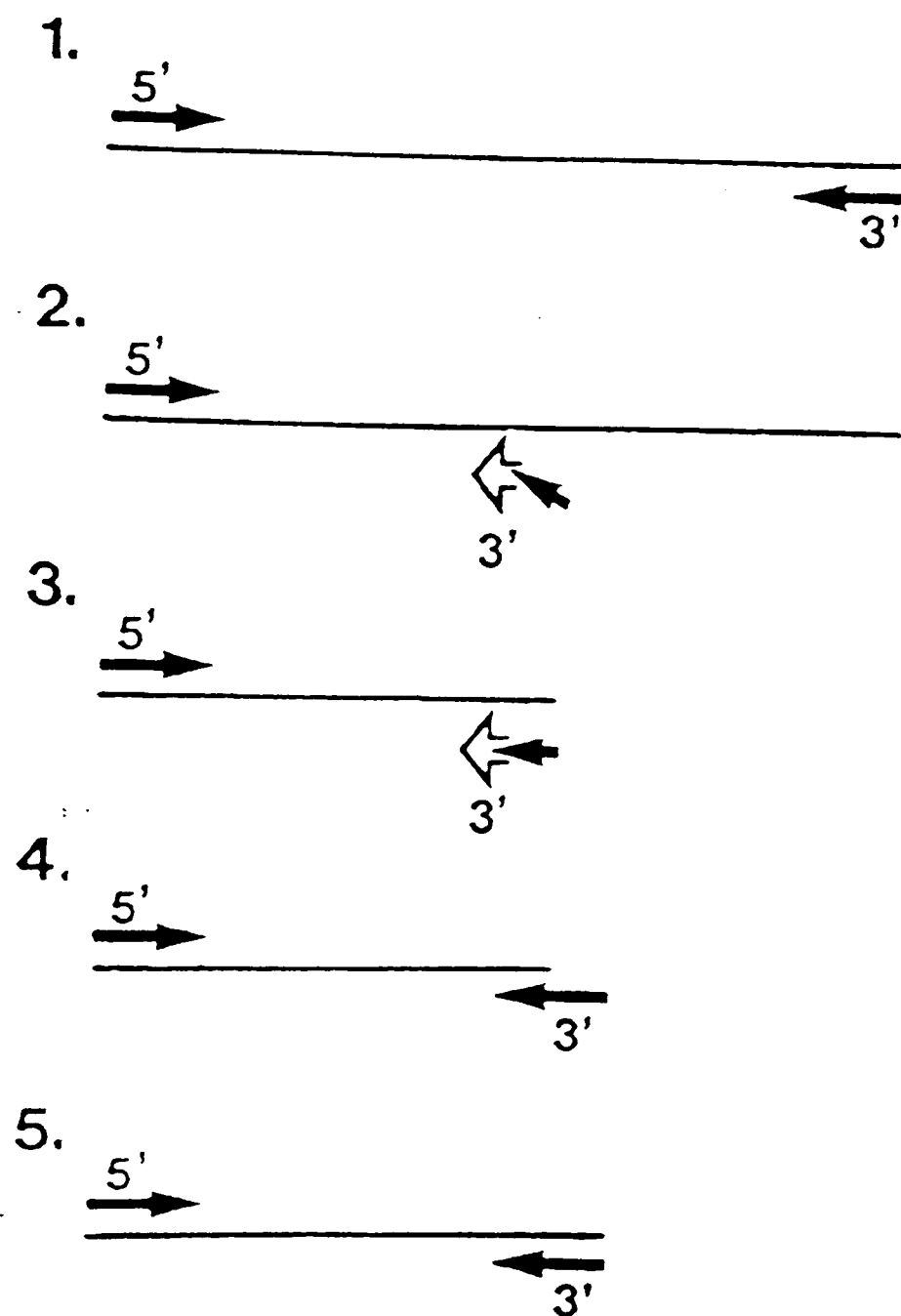


Fig 5.1.1 Schematic view of internal standard generation; the same 5'-primer is used in all amplification steps.

1. Amplification of fungal fragment, position of original 5' and 3' primer;
 2. First re-amplification step: 3' linker primer replaces 3' primer; fungal fragment as template;
 3. Smaller fragment generated with 3' linker primer and fungal fragment as template;
 4. Second re-amplification step: Original 3' primer replaces 3' linker primer; smaller fragment as template;
 5. Standard generated with the original primers, and smaller fragment as template.
- After Forster (1994).

RESULTS

When the internal standard sequences developed for *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* were diluted and amplified in the presence of a constant amount of fungal DNA using PCR, competitor bands corresponding in intensity to fungal bands could be identified for each of the species. PCR amplified internal standard dilution series are illustrated for *F.culmorum* and *M.nivale* var. *nivale* in Figs 5.1.2 and Fig 5.1.3 respectively. A similar dilution series was carried out for *M.nivale* var. *majus*. In the case of *F.culmorum*, a 0.06×10^4 dilution of the internal standard was chosen as the concentration likely to be useful over the widest range of fungal DNA concentrations. Similarly, dilutions of 1.0×10^4 and 0.5×10^3 of the internal standards were chosen for *M.nivale* var.*nivale* and *M.nivale* var.*majus*.

When these standards were amplified in the presence of a range of DNA concentrations of the relevant pathogen, it was possible to determine the range of DNA concentrations quantifiable with each of the competitors. When the concentration of fungal DNA was very low in the sample, only the competitor PCR product could be detected, whereas when the concentration of fungal DNA was high, and was able to outcompete the internal standard for the PCR primers, only the fungal PCR fragment was present. At fungal DNA concentrations between these two limits, two PCR products were detectable. The fungal dilution series for *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* are illustrated in Figures 5.1.4 b, 5.1.5 b and 5.1.6 b.

The photographic negatives of each of these fungal dilution series were scanned using densitometry, and the ratio of fungal:competitor DNA calculated by a comparison of the peak heights produced by each fragment. For each of these ratios, the amount of fungal DNA in the sample had been calculated, and a calibration of the fungal DNA concentration to the ratio of fungal:competitor DNA could therefore be plotted (see Figs 5.1.4a, 5.1.5a and 5.1.6a).

DNA from plants previously inoculated with various combinations of *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var. *majus* (see chapter 4) was analysed to quantify *F.culmorum* using a PCR assay incorporating the relevant amount of *F.culmorum* internal standard DNA. Each treatment consisted of samples of DNA from individual plants, and each had three replicates (18 plants total). The PCR products obtained from the assay to assess amounts of *F.culmorum* DNA in treatments inoculated with *F.culmorum* in one replicate of treatments 1, 4, 5, 7 and 8 are shown in Fig 5.1.7. Plant samples inoculated with *F.culmorum* produced fragments corresponding in size to those produced by *F.culmorum* fungal DNA using a conventional PCR reaction. In most cases, a competitor fragment smaller in size than the fungal DNA was also visible. In the control samples (treatment 8 - uninoculated) no fungal fragment was observed, and a competitor fragment was clear in all cases. A ratio of *F.culmorum*:competitor DNA calculated for each sample in each of the three replicates. These ratios were then converted into relative amounts of DNA (Table 5.1.1). The amount of DNA present/mg dry plant material may not be completely accurate due to problems encountered with the *F.culmorum* competitor fragment. The concentration of competitor chosen from the original dilution series was used in the plant assay, but the fungal dilution series from which the calibration was calculated was carried out at a later date using a different competitor concentration due to degradation of the original sample. However, the values of fungal DNA calculated from this calibration will still be in proportion to each other and will therefore provide an indication of the relative amounts of *F.culmorum* in each plant. Table 5.1.1 shows that no *F.culmorum* DNA could be detected in the uninoculated control treatment (treatment 8). In replicate three, most plant samples from treatments 1, 4, 5 and 7 were infected with more than 4.36µg/mg *F.culmorum* fungal DNA, whereas replicate 2 had in general less infection in all treatments, and replicate one showed very little infection in any of the inoculated treatments. There did not

appear to be any difference in the amount of *F.culmorum* DNA quantified in each of the treatments, according to the species inoculated. All treatments (except the control treatment 8) were infected with *F.culmorum* and there appeared to be a much greater effect of replicate than of treatment on the amount of *F.culmorum* DNA present.

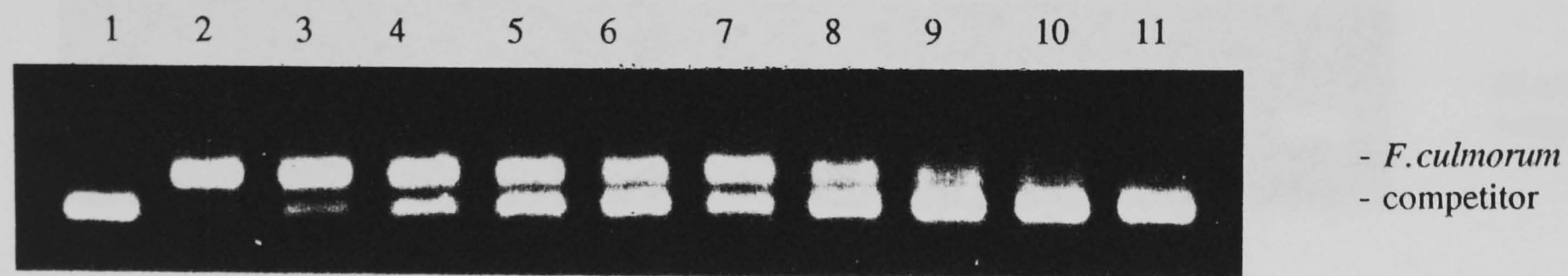


Fig 5.1.2 PCR products obtained from the amplification of a constant amount of *F. culmorum* fungal DNA in the presence of varying dilutions of the *F. culmorum* competitor fragment. Lane 1 = competitor DNA control, 2 = 0.06×10^4 dilution of competitor, 3 = 0.125×10^4 , 4 = 0.25×10^4 , 5 = 0.5×10^4 , 6 = 1×10^4 , 7 = 0.06×10^3 , 8 = 0.125×10^3 , 9 = 0.25×10^3 , 10 = 0.5×10^3 , 11 = 1×10^3 .

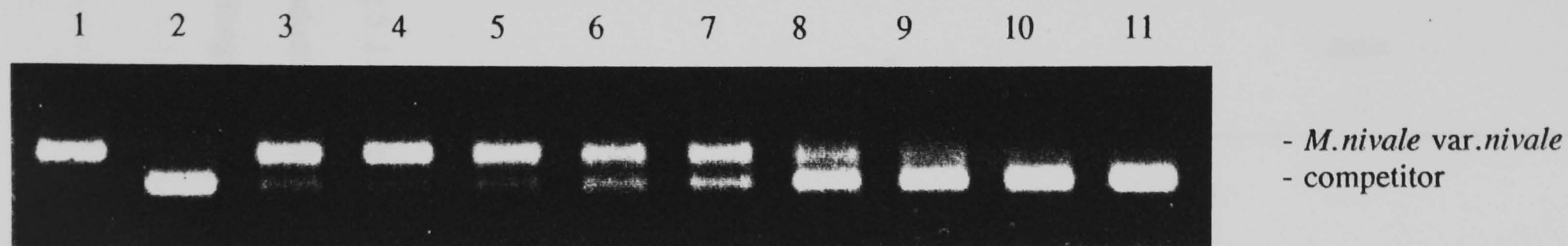


Fig 5.1.3 PCR products obtained from the amplification of a constant amount of *M.nivale* var.*nivale* fungal DNA in the presence of varying dilutions of the var.*nivale* competitor fragment. Lane 1 = *M.nivale* var.*nivale* fungal DNA control., 2 = *M.nivale* var.*nivale* competitor DNA control, , 3 = 0.125×10^4 , 4 = 0.25×10^4 , 5 = 0.5×10^4 , 6 = 1×10^4 , 7 = 0.06×10^3 , 8 = 0.125×10^3 , 9 = 0.25×10^3 , 10 = 0.5×10^3 , 11 = 1×10^3 dilution competitor.

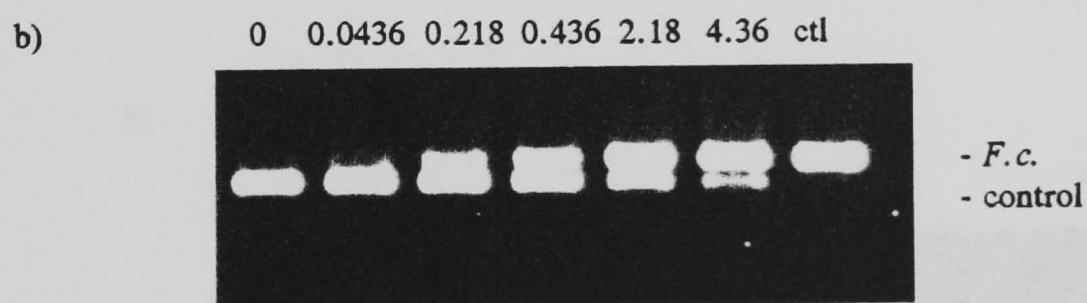
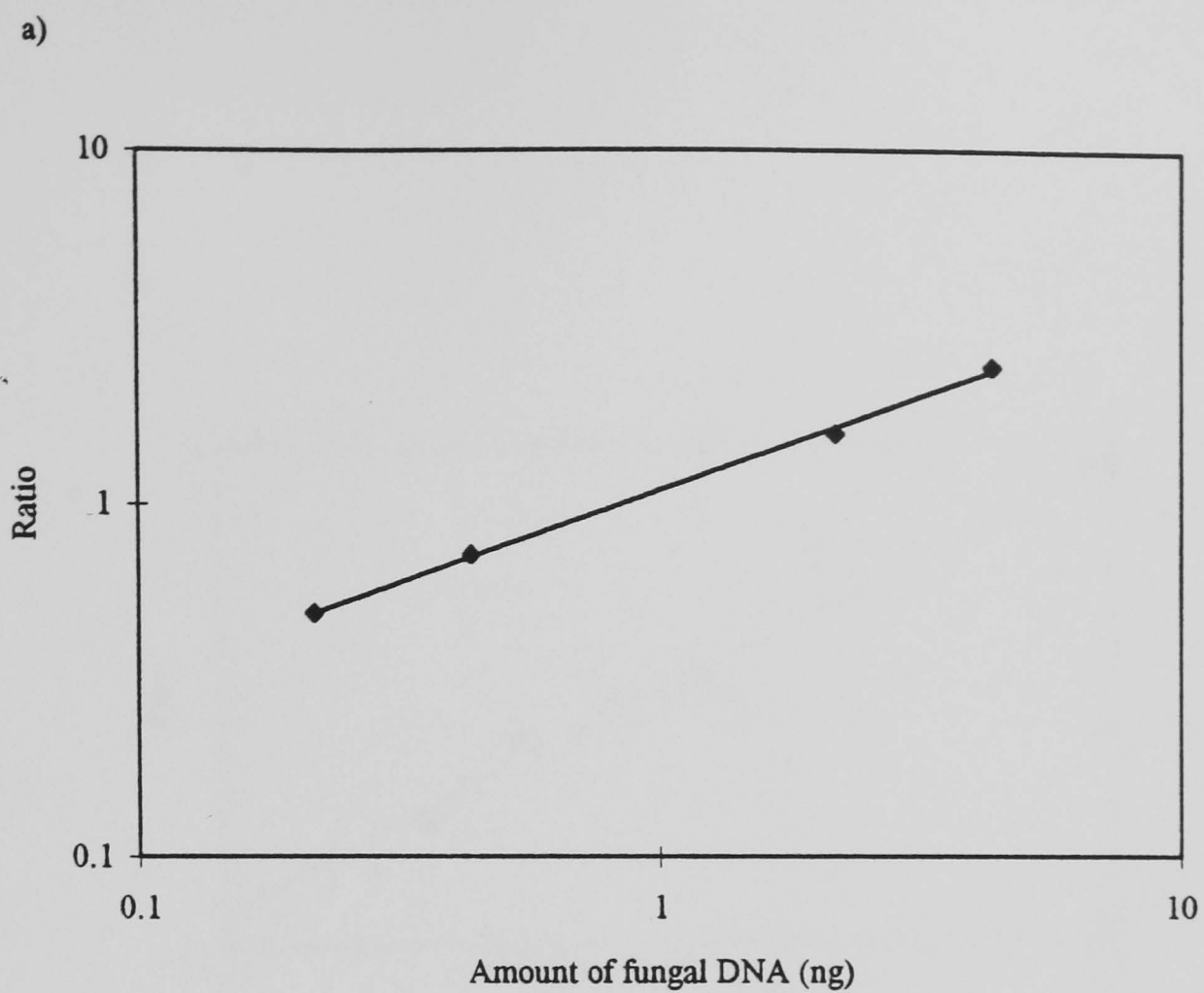
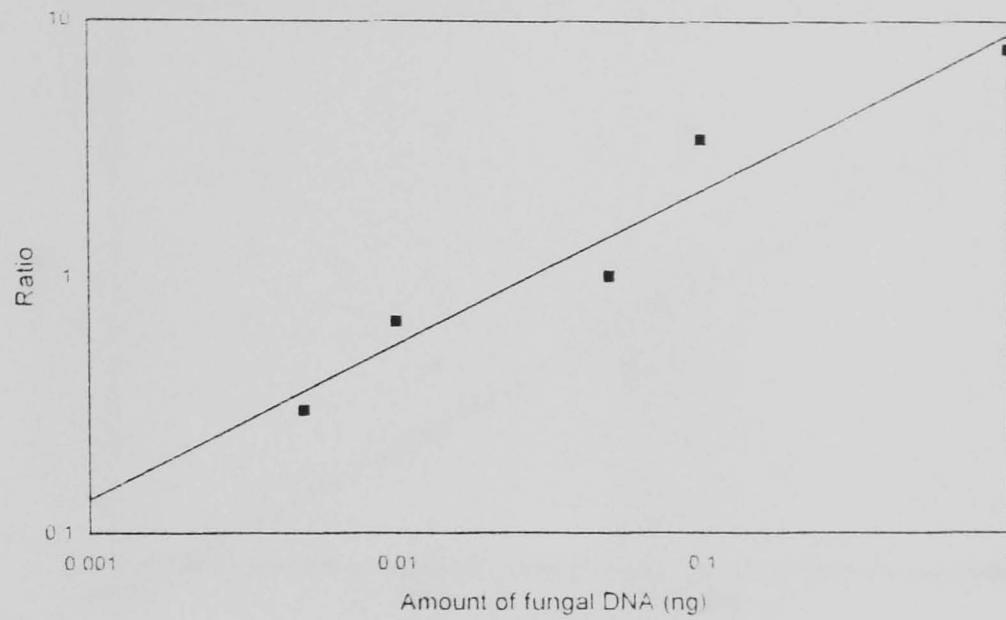


Fig 5.1.4 Relationship between the amount of DNA of *Fusarium culmorum* (*F.c.*) and PCR product ratio a) derived from densitometer readings of reactions containing 0.0436-4.36ng of fungal DNA in the presence of a constant amount of competitor template DNA b)

a)



b)

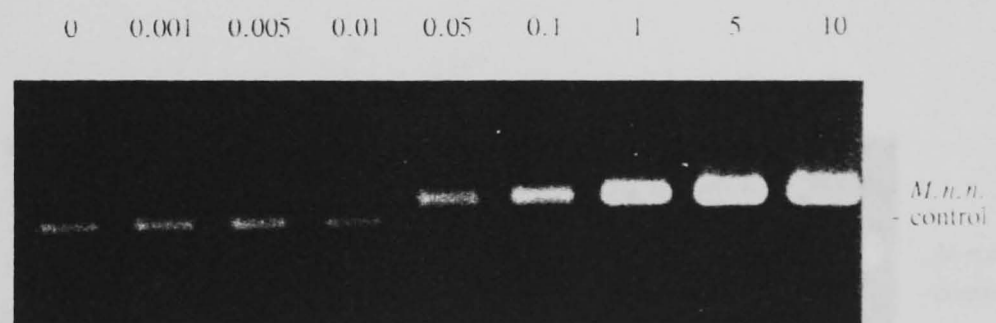
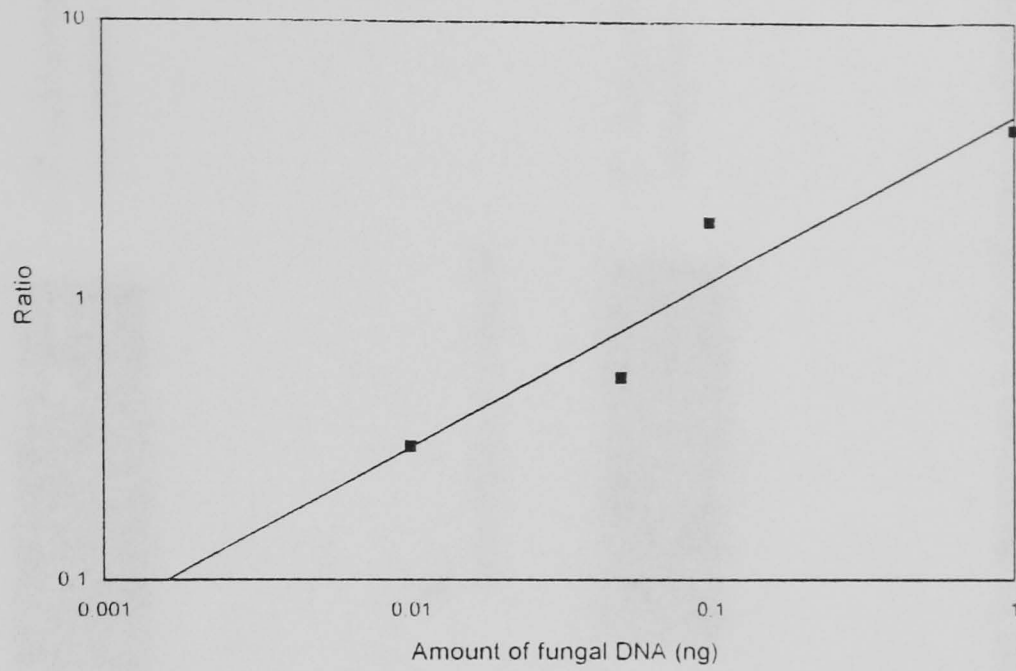


Fig 5.1.5 Relationship between the amount of DNA of *Microdochium nivale* var.*nivale* (*M.n.n*) and PCR product ratio a) derived from densitometer readings of reactions containing 0.001-10ng of fungal DNA in the presence of a constant amount of competitor template DNA b).

a)



b)

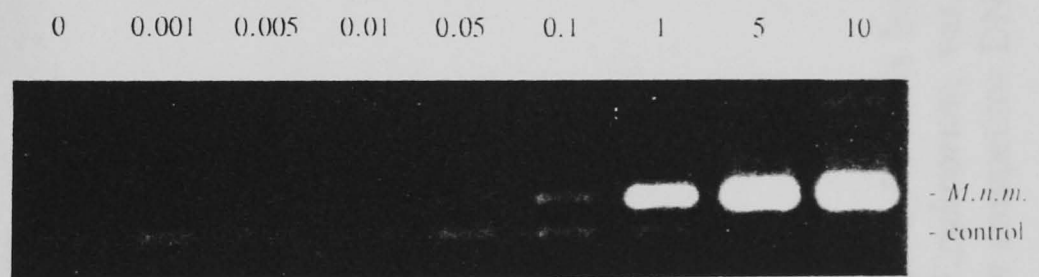


Fig 5.1.6 Relationship between the amount of DNA of *Microdochium nivale* var. *majus* (*M.n.m*) and PCR product ratio a) derived from densitometer readings of reactions containing 0.001-10ng of fungal DNA in the presence of a constant amount of competitor template DNA b).

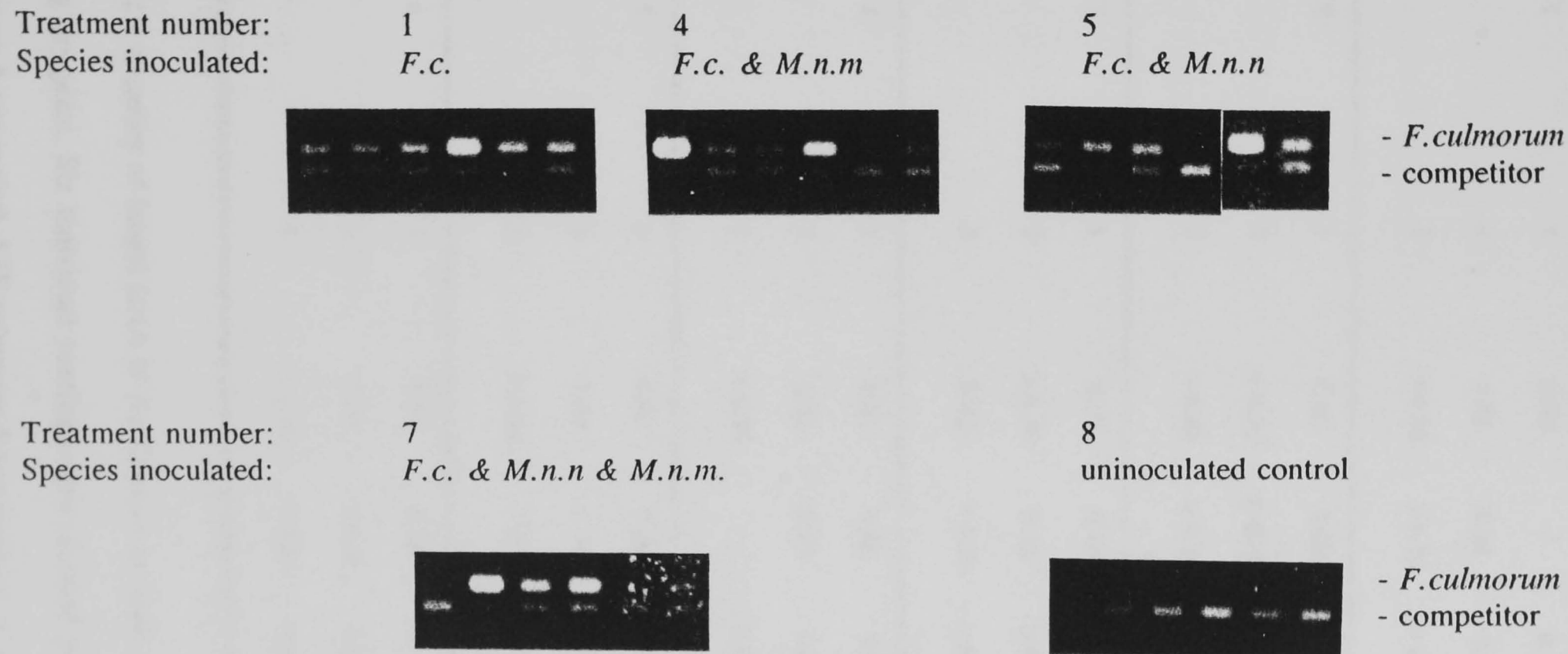


Fig 5.1.7 Amplification products of DNA obtained from six individual plants in treatments 1 (*F.culmorum* inoculated), 4 (*F.culmorum* & var.*majus*), 5 (*F.culmorum* & var.*nivale*), 7 (*F.culmorum*, var.*majus* and var.*nivale*) and 8 (uninoculated), amplified using PCR in the presence of a known amount of the *F.culmorum* competitor DNA fragment. *Fusarium culmorum* = *F.c.*, *M.nivale* var.*nivale* = *M.n.n.*, *M.nivale* var.*majus* = *M.n.m.*

Seedling number	Replicate number	Treatment number				
		1	4	5	7	8
1	1	0.00	-	0.37	0.00	0.00
	2	1.89	0.34	0.04	0.00	0.00
	3	>4.36	>4.36	>4.36	>4.36	0.00
2	1	0.00	0.00	0.61	0.00	0.00
	2	>4.36	0.0075	1.55	0.00	0.00
	3	>4.36	>4.36	>4.36	>4.36	0.00
3	1	0.00	0.45	0.00	0.00	0.00
	2	>4.36	0.34	>4.36	>4.36	0.00
	3	0.00	>4.36	>4.36	>4.36	0.00
4	1	0.00	0.00	0.00	0.00	0.00
	2	2.54	0.69	0.25	0.09	0.00
	3	>4.36	-	>4.36	>4.36	0.00
5	1	0.00	0.00	0.00	0.00	0.00
	2	2.69	0.79	0.91	2.52	0.00
	3	>4.36	1.01	>4.36	>4.36	0.00
6	1	0.20	0.00	0.00	0.00	0.00
	2	1.70	>4.36	>4.36	>4.36	0.00
	3	>4.36	>4.36	>4.36	>4.36	0.00

Table 5.1.1 Quantity of fungal DNA of *F.culmorum* in stem base tissue expressed as ng fungal DNA/mg dry plant. Six individual seedlings were assessed from treatments 1 (*F.culmorum*), 4 (*F.culmorum* & var.*majus*), 5 (*F.culmorum* & var.*majus*) 7 (*F.culmorum*, var.*nivale* & var.*majus*) and 8 (uninoculated) and each seedling had three replicates. - indicates reaction failure.

DISCUSSION

It was demonstrated that quantification of fungal DNA from *F.culmorum*, *M.nivale* var. *nivale* and *M.nivale* var. *majus* was possible using an adapted PCR assay incorporating an internal standard control fragment. The development of these assays using the method of Forster (1994) allowed the rapid generation of the competitor fragment by PCR, where only a knowledge of sequence information for each of the pathogens was required.

The amplification of a range of concentrations of the competitor DNA fragments from each pathogen in the presence of a constant amount of the relevant fungal DNA provided a simple method of selection of the concentration likely to be useful for the measurement of fungal DNA. Despite problems with the further PCR amplification of the chosen concentration of competitor fragment with a range of known fungal DNA concentrations in the case of *F.culmorum*, this test ensured that the competitor would be able to compete for the PCR primers in the presence of a range of concentrations of fungal DNA, and provided a method for the calibration of the system. Fungal DNA of *M.nivale* var. *nivale* and *M.nivale* var. *majus* was quantifiable in the range 0.001 - 1ng DNA/mg of dry material, and in the range 0.1-10ng/mg in the case of *F.culmorum*. Problems could be avoided in further tests by carrying out all calibrations and assays within a reasonable time of one another, and by purification of the competitor DNA fragment to avoid degradation. The method of comparing the ratio of fungal:competitor DNA using densitometry for calibration was effective, and avoided the necessity to use radiolabelling, as opposed to those quantification systems described by other workers (Hayashi *et al.*, 1989, Murphy *et al.*, 1990, Hu *et al.*, 1993). Porcher *et al* (1993) modified a method of quantification involving the use of internal standards, as described in this chapter, by the addition of fluorescently labelled PCR primers. The PCR products obtained are loaded onto an automatic DNA sequencer, and submitted to

electrophoresis. The ratio of the peaks corresponding to the target and competitor fragments can then be calculated to determine the target DNA concentration. The method described by Porcher *et al.* (1993) appears to be appropriate for use with the quantification system developed in this work, and should provide an improved method of quantification of *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus*.

Competitive PCR provides a good indication of reaction failure: in conventional PCR, the absence of a certain product is usually interpreted as an absence of the original target DNA, the amplification of the competitor band in samples where no fungal DNA is present therefore provides a reliable indicator of reaction failure. Work has shown (Bej *et al.*, 1992) that PCR reactions can be inhibited by the presence of humic material in samples. In samples obtained from plants where contamination with humic substances is not unlikely, the presence of a competitor fragment to indicate reaction failure is therefore particularly useful.

When individual plant samples were assessed for infection by *F.culmorum* using competitive PCR, it was found that there were very large differences in the amount of fungal DNA present between replicates. This level of accuracy would not have been visible using conventional species specific PCR, where each sample would only have been scored according to the presence or absence of the fragment. The reasons for these discrepancies between replicates are not known, but may have been due to environmental conditions in replicates being different, and therefore affecting the infection process by *F.culmorum*. There did not appear to be any differences between the amount of *F.culmorum* detected in each of the different treatments, apart from the uninoculated treatment, where no fungal DNA was detected, although it is possible that the effects of competition between the three pathogens inoculated may have been hidden by the large replicate differences. In order to investigate competition between species further, it would be necessary to conduct a far larger test to quantify the amount

of each pathogen in more individual plants, and to increase the number of replicates. This work has shown the development of a quantitative PCR assay for *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus*. Further work can now be carried out using these tools to investigate the interaction between foot rot pathogens under varying environmental conditions, the relationship between disease symptom severity and infection, the effects of fungicides on each of the pathogens, and possibly the resistance of plants to infection by these pathogens.

CONCLUSIONS

It was shown that although the triazole fungicide flusilazole, and the imidazole fungicide prochloraz were able to control the germination of conidia and growth of *F.culmorum*, *F.avenaceum* and *M.nivale in vitro* to a varying extent, no consistent control of these pathogens was observed in the field using the same fungicides when applied to wheat. Similarly, no differences in plant emergence or establishment were observed in an experiment to investigate the efficacy of an organomercury seed treatment and a pyrrolecarbonitrile seed treatment in reducing emergence and establishment of winter wheat caused by these pathogens. In further field and semi-controlled environment experiments (Chapter 2), the fungicide fludioxinil was also shown to be ineffective in controlling stem base disease caused by *F.culmorum*, *F.avenaceum* and *M.nivale*, and there were no increases in yield in the treated plots. Reasons for this lack of field control of pathogens by fungicides could be manifold, and are further discussed in the relevant Chapters. In these field experiments, many difficulties were encountered with symptom identification, the isolation of pathogens from plant material and the subsequent identification of species isolated. However, results from this work have shown that infection of winter wheat by *F.culmorum* and *M.nivale* outdoors under semi-controlled environmental conditions reduces the yield of wheat, (Chapter 2). Yield losses were greatest when inoculum density was high and infection occurred early in the season.

This field work highlighted the requirement for a more accurate method of pathogen detection in plant tissue, and illustrated the problems associated with conventional isolation and identification techniques. Species specific PCR primers were developed for the detection of *M.nivale* var.*nivale*, *M.nivale* var.*majus*, *F.culmorum*, and *F.avenaceum* in plant tissue. The development of this detection system (see Chapter 4) allowed the rapid and accurate detection and identification of these pathogens in host

plant material, and therefore avoided the necessity of lengthy and sometimes inaccurate isolation and identification of the pathogens using conventional techniques. It is now therefore possible to apply this system to investigate the effects of fungicides, the competition between species on infection of wheat plants, and the epidemiology of the pathogens. As species specific primers have also been developed for W and R-type eyespot (Nicholson & Rezanoor 1994), *Rhizoctonia cerealis* (Nicholson pers. comm.) and *Fusarium poae* (Parry pers. comm.), it is also possible to study the impact of extraneous factors on individual pathogens occurring alone or as a complex in stem base and ear diseases. Although no obvious interactions between pathogens were noted in competition experiments carried out (Chapter 4), further work is required in order to investigate the effect of competition between the stem base pathogens.

It was also possible, using molecular techniques including RAPDs to elucidate the taxonomy of *Microdochium nivale*, and to provide evidence that a distinct sub-group of this pathogen could be identified (Lees *et al.*, 1995). In a survey of winter wheat crops in England in 1994, Parry *et al.* (1995), using species specific PCR, showed that two types of *M.nivale* could be identified which corresponded to the sub-groups *M.nivale* var.*nivale* and *M.nivale* var.*majus* as suggested by Lees *et al.* (1995).

A further development of PCR detection of pathogens was the employment of competitive PCR for the quantification of fungal infections in plant material, (Chapter 5). In this work a quantification system based on the competition between fungal DNA of either *F.culmorum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus* and a competitor DNA fragment derived from the same species in a PCR assay was developed. By a comparison of the ratio of fungal:competitor PCR product using densitometry, these pathogens could be quantified. This ability to quantify pathogens in plant tissue will allow more accurate studies of stem base disease pathogen interactions, effects of fungicides and epidemiology to take place.

In conclusion, the work presented here has provided tools for the further examination of the stem base disease complex of wheat. Initial problems with the identification and detection of *Fusarium culmorum*, *Fusarium avenaceum* and *M.nivale* in wheat stem base tissue highlighted in the field experiments described in Chapters 1 and 2 have been overcome by the development of sensitive species specific primers able to detect each of these pathogens in wheat tissue in a PCR reaction. A more accurate examination of the relationship between fungal infection by these pathogens, disease symptom severity, yield loss and fungicide performance will therefore now be possible using these tools. Other areas of investigation facilitated by the development of these tools include epidemiological studies of each of these pathogens, studies of the competition between and pathogenicity of each of these species, and assessments of varietal resistance to disease. The development of the quantitative PCR assay described in Chapter 5 will be particularly useful for studies of this type where accurate estimations of fungal biomass are necessary.

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APPENDICES

Appendix 1.1.1 SEM values of Fig 1.1

		Concentration of flusilazole (µg/ml a.i.)					
		0	0.05	0.1	1	10	50
Time (hrs)	0	0	0	0	0	0	0
	3	0	3.03	0.85	0.48	0	0
	6	0.25	0.85	1.11	2.2	1	0
	9	0.48	0.48	0.48	0.75	0	0
	12	0	0	0	0	0	0
	24	0	0	0	0	0	0

Appendix 1.1.2 SEM values of Fig 1.2

		Concentration of prochloraz (µg/ml a.i.)					
		0	0.05	0.1	1	10	50
Time (hrs)	0	0	0	0	0	0	0
	3	2.12	0.78	0.92	1.14	0.12	0.4
	6	1.29	0.95	0.42	0.36	1.15	1.15
	9	0.68	0.97	0.71	0.82	0.4	0
	12	0	0.31	1.11	1.11	0	0.4
	24	0	0	0.36	0.34	0	0

Appendix 1.1.3 SEM values of Fig 1.3

		Concentration of fungicide (µg/ml a.i.)					
		0	0.05	0.1	1	10	50
Flusilazole		2.74	1.53	1.61	1.6	0	0
Prochlora		1.56	0.98	1.03	1	0	0

Appendix 1.1.4 SEM values of Fig 1.4

		Concentration of flusilazole (µg/ml a.i.)					
		0	0.1	1	10	50	
Time (hrs)	0	0	0	0	0	0	
	2	1.5	0.3	0.95	0.22	0	
	3	1.5	0.68	0.74	0.7	0.16	
	7	0	2.42	2.21	0	1.52	
	8	0	4.5	2.52	1.12	1.79	
	9	0	3.83	2.09	1.05	1.68	

Appendix 1.1.5 SEM values for Fig 1.5

		Concentration of prochloraz (µg/ml a.i.)					
		0	0.1	1	10	50	
Time (days)	0	0	0	0	0	0	
	2	1	0.64	1.23	0	0	
	3	1.7	0.54	1.87	0	0	
	7	0	0.42	2.4	0	0	
	8	0	0.98	2.72	0	0	
	9	0	2.52	2.04	0	0	

Appendix 1.1.6 SEM values for Fig 1.6

		Concentration of fungicide (µg/ml a.i.)					
		0	0.1	1	2	10	50
flusilazole		0	1.34	3.32	2.44	2.33	0
prochloraz		0	2.27	2.03	8.44	0	0

Appendix 1.1.7 SEM values for Fig 1.7

Time (hrs)	Concentration of flusilazole (µg/ml a.i.)					
	0	0.05	0.1	1	10	50
0	0	0	0	0	0	0
3	0.25	0	0	0.25	0.25	0
6	0	0	0	0	0	0
9	0.67	0.72	1.12	1.74	0.89	0.67
12	1.28	1.03	1.07	0.56	0.76	0.56
24	0	0	0	0	0	0

Appendix 1.1.8 SEM values for Fig 1.8

Time (hrs)	Concentration of prochloraz (µg/ml a.i.)					
	0	0.05	0.1	1	10	50
0	0	0	0	0	0	0
3	0	0	0	0	0	0
6	0	0	0	0	0	0
9	0.13	0.42	0.62	0.79	1.14	0
12	0.43	0.98	0.74	1.71	1.08	0.72
24	0	0.74	0.92	2.13	2.04	1.67

Appendix 1.1.9 SEM values of Fig 1.9

	Concentration of fungicide (µg/ml a.i.)					
	0	0.05	0.1	1	10	50
flusilazole	0.56	1.03	0.72	0.64	0.76	0.58
prochloraz	1.01	0.58	0.69	0.74	0.58	0.72

Appendix 1.1.10 SEM values of Fig 1.10

Time (days)	Concentration of flusilazole (µg/ml a.i.)				
	0	0.1	1	10	50
0	0	0	0	0	0
2	1.51	0.84	0.71	0	0
3	1.39	0.71	1.43	0.26	0
7	0	0.79	3.43	0	0
8	0	0	3.23	0	0
9	0	0	5.58	0	0

Appendix 1.1.11 SEM values of Fig 1.11

Time (days)	Concentration of prochloraz (µg/ml a.i.)				
	0	0.1	1	10	50
0	0	0	0	0	0
2	1.51	0.26	0	0	0
3	1.39	0.63	0	0	0
7	0	0.84	0	0	0
8	0	1.03	0	0	0
9	0	0.39	0	0	0

Appendix 1.1.12 SEM values of Fig 1.12

	Concentration of fungicide (µg/ml a.i.)					
	0	0.1	1	2	10	50
flusilazole	0	1.98	1.4	1.04	0	0
prochloraz	0	0.47	0	0	0	0

Appendix 1.1.13 SEM values for Fig 1.13

Time (hrs)	Concentration of flusilazole (µg/ml a.i.)					
	0	0.05	0.1	1	10	50
0	0	0	0	0	0	0
3	0	0	0	0	0	0
6	1.32	1.26	1	1.15	0.76	0.5
9	0.76	1	0.76	0.76	1.76	2.02
12	1.52	1.61	1.04	1.26	0.29	1.8
24	0	0	0	0	0	0

Appendix 1.1.14 SEM values for Fig 1.14

Time (hrs)	Concentration of prochloraz (µg/ml a.i.)					
	0	0.05	0.1	1	10	50
0	0	0	0	0	0	0
3	0	0	0	0	0	0
6	0.96	0.21	1.14	0.76	2.14	0.21
9	0.72	0.36	2.22	0.98	0.75	0.26
12	0.31	0.42	0.79	1.72	0.87	0.72
24	0	0.31	1.03	0.14	0.42	0.41

Appendix 1.1.15 SEM values of Fig 1.15

	Concentration of fungicide (µg/ml a.i.)					
	0	0.05	0.1	1	10	50
flusilazole	0.63	0.67	0.67	0.66	0.57	0.42
prochloraz	0.92	0.85	0.47	0.69	0.58	0.63

Appendix 1.1.16 SEM values of Fig 1.16

Time (days)	Concentration of flusilazole (µg/ml a.i.)				
	0	0.1	1	10	50
0	0	0	0	0	0
2	0.92	0.84	0.26	0.26	0
3	1.13	0.84	0.91	0.33	0
7	1.98	0.86	1.02	1.2	0.91
8	0.82	0.83	2.67	1.22	0.86
9	0	1.23	2.93	1.52	1.79

Appendix 1.1.17 SEM values of Fig 1.17

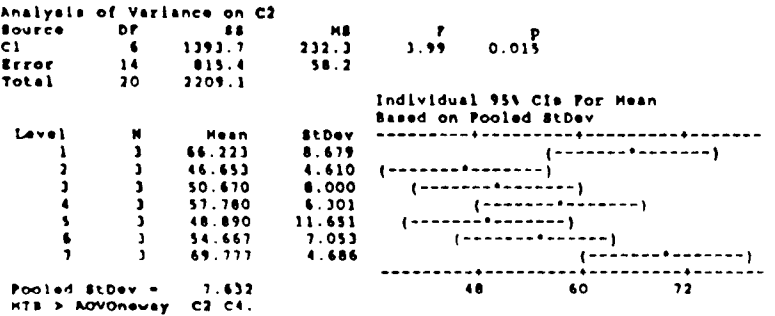
Time (days)	Concentration of prochloraz (µg/ml a.i.)				
	0	0.1	1	10	50
0	0	0	0	0	0
2	0.91	0.21	0	0	0
3	1.13	1.17	0	0	0
7	2.71	2.17	0	0	0
8	3.25	2.24	0	0	0
9	0	2.34	0	0	0

Appendix 1.1.18 SEM values of Fig 1.18

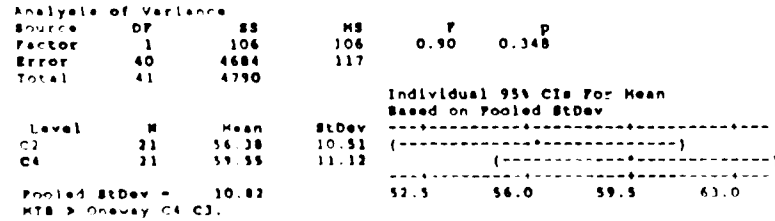
	Concentration of fungicide (µg/ml a.i.)					
	0	0.1	1	2	10	50
flusilazole	2.43	2.43	1.53	1.11	0.65	0.94
prochloraz	0	1.73	0	0	0	0

Appendix 2.1.1 (corresponding to Fig 2.1.1)

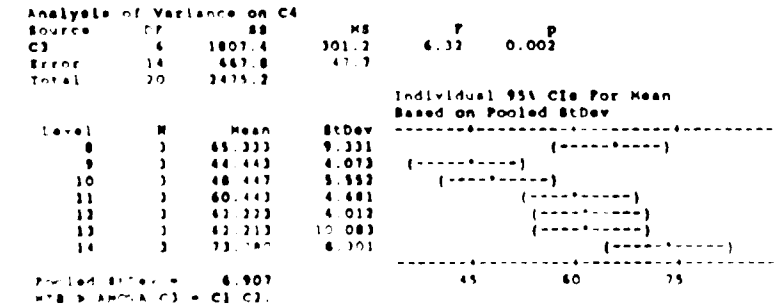
One-Way Analysis of Variance



One-Way Analysis of Variance



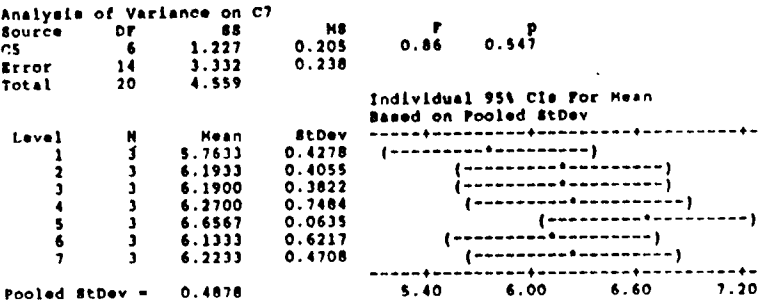
One-Way Analysis of Variance



MTB >

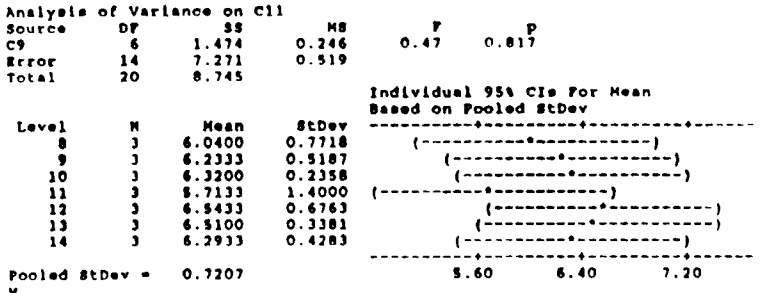
Appendix 2.1.3 (corresponding to Fig 2.1.3)

One-Way Analysis of Variance



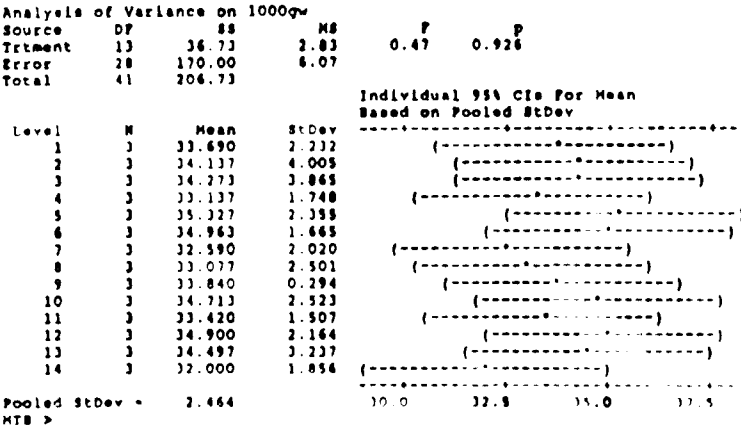
MTB > Oneway C11 C9.

One-Way Analysis of Variance



Appendix 2.1.4 (corresponding to Fig 2.1.4)

One-Way Analysis of Variance



Appendix 2.3.1 (corresponding to Fig 2.3.1)

comparison	p value (from students t-test)
0/1	0.0232
0/2	0.00281
0/3	3.8×10^{-4}

One-Way Analysis of Variance

Analysis of Variance					
Source	DF	SS	MS	F	P
Factor	3	16854	5618	7.96	0.000
Error	116	81874	706		
Total	119	98728			

Level	N	Mean	StDev
marcul0	30	4.80	9.97
marcul1	30	13.53	24.38
marcul2	30	26.10	28.73
marcul3	30	35.87	36.11

Individual 95% CIs For Mean
Based on Pooled StDev

Appendix 2.3.2 (corresponding to Fig 2.3.2)

comparison	p value (from students t-test)
0/1 Mar	0.0039
0/2 Mar	0.0022
0/3 Mar	4.6×10^{-10}
comparison	p value (from students t-test)
0/1 May	0.000001
0/2 May	4.35×10^{-14}
0/3 May	4.88×10^{-14}
comparison	p value (from students t-test)
0/1 Jun	0.043
0/2 Jun	0.15
0/3 Jun	0.043

Analysis of Variance					
Source	DF	SS	MS	F	P
Factor	3	66239	22080	24.88	0.000
Error	116	102927	887		
Total	119	169166			

Level	N	Mean	StDev
2marcul0	30	12.80	23.02
2marcul1	30	39.60	30.64
2marcul2	30	68.00	34.73
2marcul3	30	70.33	29.56

Pooled StDev = 29.79

Individual 95% CIs For Mean
Based on Pooled StDev

Analysis of Variance					
Source	DF	SS	MS	F	P
Factor	3	70823	23608	30.79	0.000
Error	116	88949	767		
Total	119	159771			

Level	N	Mean	StDev
2aprcul3	30	96.60	32.98
2aprcul2	30	67.00	24.20
2aprcul1	30	47.67	26.32
2aprcul0	30	31.33	26.49

Pooled StDev = 27.69

MTB > AOVOneway '2maycul0' '2maycul1' '2maycul2' '2maycul3'.

Individual 95% CIs For Mean
Based on Pooled StDev

Appendix 2.3.3 (corresponding to Fig 2.3.3)

comparison	p value (from students t-test)
0/1 Mar	0.011
0/2 Mar	0.0067
0/3 Mar	0.0033
comparison	p value (from students t-test)
0/1 May	0.036
0/2 May	0.22
0/3 May	7.96×10^{-1}
comparison	p value (from students t-test)
0/1 Jun	0.184
0/2 Jun	0.32
0/3 Jun	0.155

Analysis of Variance					
Source	DF	SS	MS	F	P
Factor	3	28.717	9.572	26.05	0.000
Error	8	2.940	0.367		
Total	11	31.657			

Level	N	Mean	StDev
culmar0	3	9.643	0.245
culmar1	3	6.567	0.804
culmar2	3	5.697	0.772
culmar3	3	6.163	0.409

Pooled StDev = 0.606

Individual 95% CIs For Mean
Based on Pooled StDev

Analysis of Variance					
Source	DF	SS	MS	F	P
Factor	3	19.792	6.597	11.22	0.003
Error	8	4.703	0.588		
Total	11	24.496			

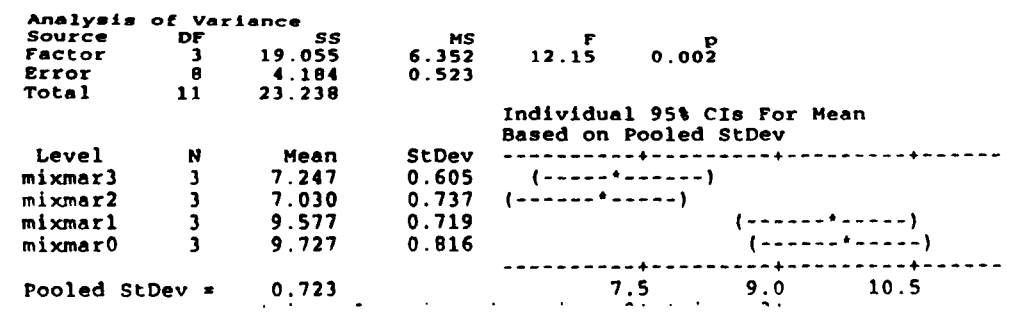
Level	N	Mean	StDev
culapr3	3	6.570	0.098
culapr2	3	7.860	1.350
culapr1	3	10.107	0.711
culapr0	3	8.683	0.119

Pooled StDev = 0.767

Individual 95% CIs For Mean
Based on Pooled StDev

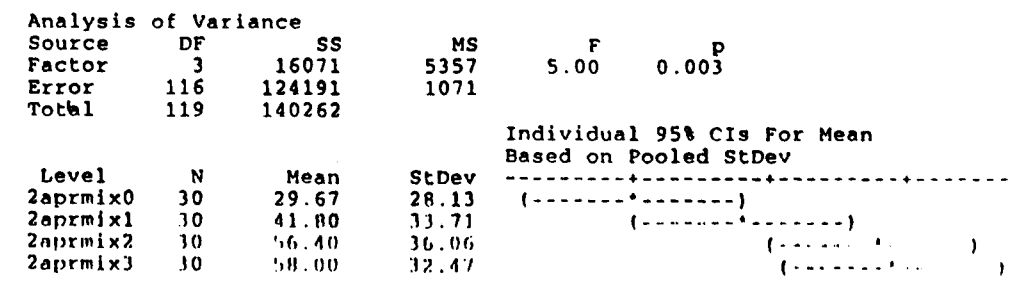
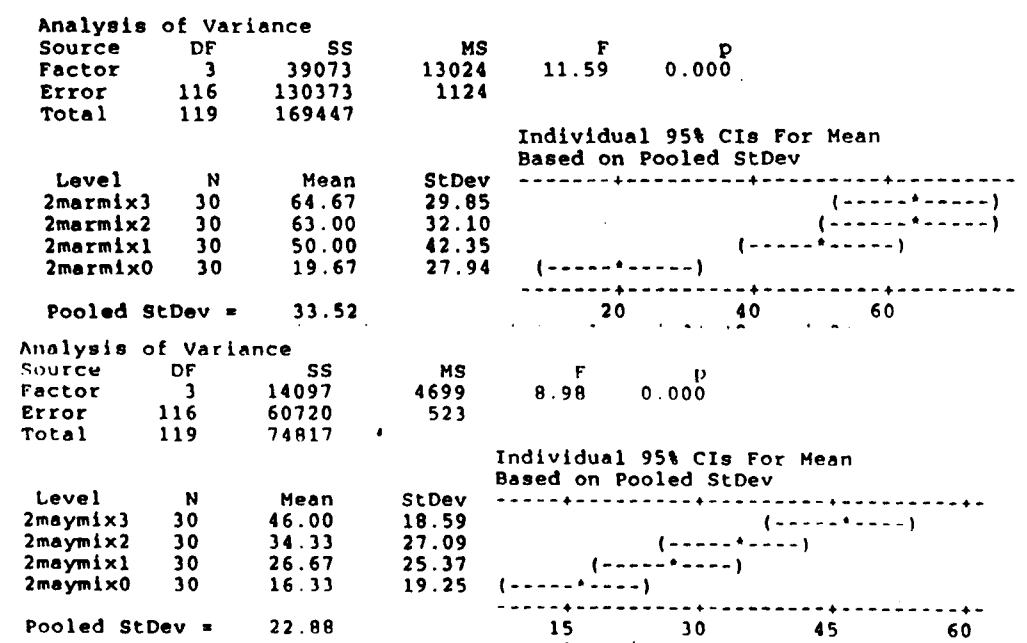
Appendix 2.3.4 (corresponding to Fig 2.3.4)

comparison	p value (from students t-test)
0/1	1.19×10^{-3}
0/2	1.71×10^{-4}
0/3	1.5×10^{-7}



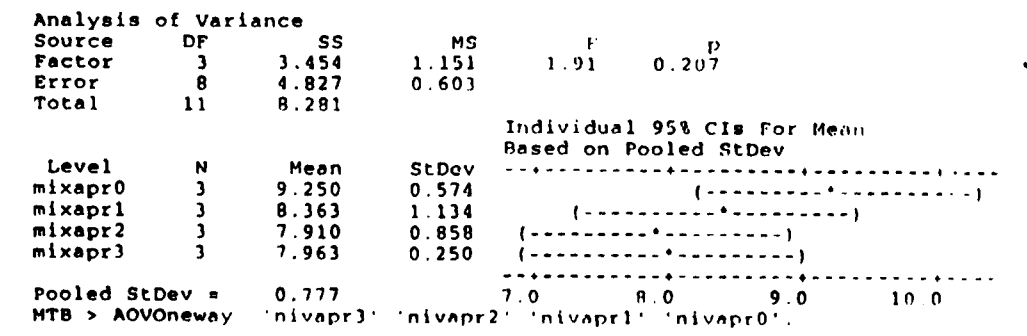
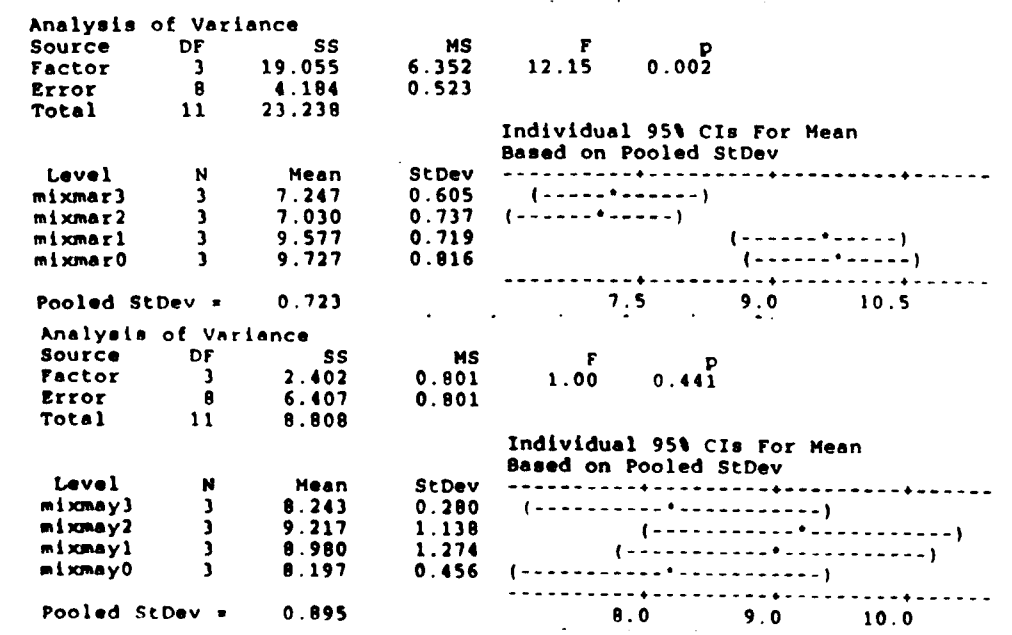
Appendix 2.3.5 (corresponding to Fig 2.3.5)

comparison	p value (from students t-test)
0/1 Mar	1.2×10^{-3}
0/2 Mar	1.46×10^{-4}
0/3 Mar	4.6×10^{-7}
comparison	p value (from students t-test)
0/1 May	0.0004
0/2 May	0.000023
0/3 May	1.72×10^{-3}
comparison	p value (from students t-test)
0/1 Jun	0.023
0/2 Jun	0.022
0/3 Jun	0.0037



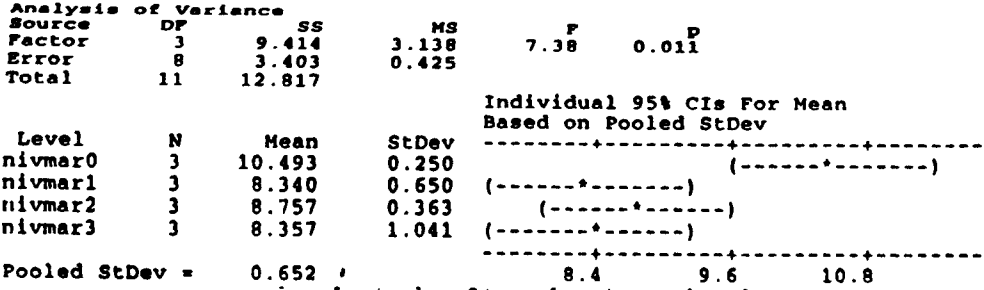
Appendix 2.3.6 (corresponding to Fig 2.3.6)

comparison	p value (from students t-test)
0/1 Mar	0.43
0/2 Mar	0.018
0/3 Mar	0.026
comparison	p value (from students t-test)
0/1 May	0.23
0/2 May	0.12
0/3 May	0.051
comparison	p value (from students t-test)
0/1 Jun	0.122
0/2 Jun	0.084
0/3 Jun	0.39



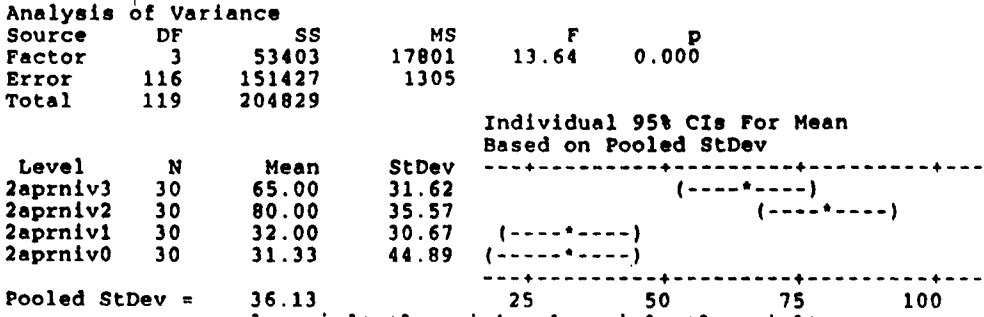
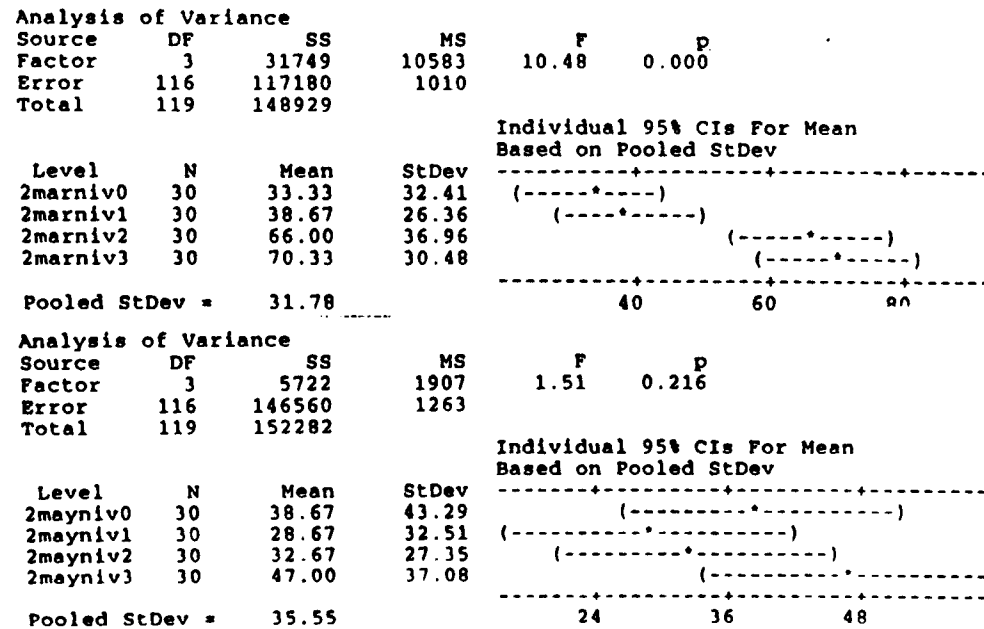
Appendix 2.3.7 (corresponding to Fig 2.3.7)

comparison	p value (from students t-test)
0/1	0.101
0/2	0.004
0/3	0.000233



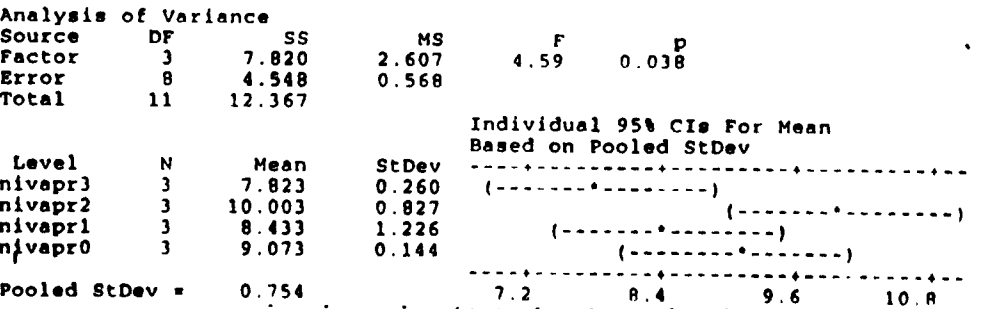
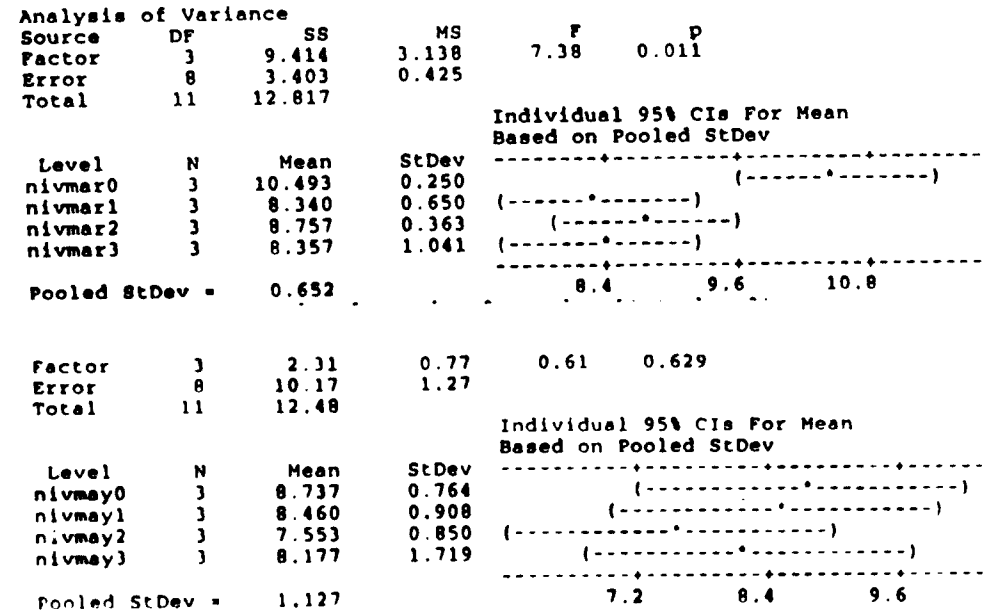
Appendix 2.3.8 (corresponding to Fig 2.3.8)

comparison	p value (from students t-test)
0/1 Mar	0.0725
0/2 Mar	0.0012
0/3 Mar	0.00026
comparison	p value (from students t-test)
0/1 May	0.21
0/2 May	0.0016
0/3 May	2.0 x 10 ⁻³
comparison	p value (from students t-test)
0/1 Jun	0.074
0/2 Jun	0.056
0/3 Jun	0.127



Appendix 2.3.9 (corresponding to Fig 2.3.9)

comparison	p value (from students t-test)
0/1 Mar	0.003
0/2 Mar	0.00071
0/3 Mar	2.7 x 10 ⁻³
comparison	p value (from students t-test)
0/1 May	0.074
0/2 May	0.14
0/3 May	0.0096
comparison	p value (from students t-test)
0/1 Jun	0.079
0/2 Jun	0.142
0/3 Jun	0.14



Appendix 4.1.1 Operon 10 base primer sequences used for the identification of *F.culmorum*, *F.avenaceum*, *M.nivale* var.*nivale* and *M.nivale* var.*majus*.

OPU08 - 5' GGCGAAGGTT 3'

OPU11 - 5' AGACCCAGAG 3'

OPU17 - 5' ACCTGGGGAG 3'

OPU19 - 5' GTCAGTGCGG 3'

OPW03 - 5' GTCCGGAGTG 3'

OPW19 - 5' CAAAGCGCTC 3'

OPY07 - 5' AGAGCCGTCA 3'

OPY13 - 5' GGGTCTCGGT 3'

OPY14 - 5' GGTCGATCTG 3'

OPY15 - 5' AGTCGCCCTT 3'